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

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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 γ2III4 is modified in γ2II14 by replacing both the central Asn and Val by Ser. Changing these replacements to Asn and Val by Ser. demonstrates an EGF-like motif γ2III4 which showed 77% identity to the nidogen binding motif γ1III4. In 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 squamous 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.

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 more 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.

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

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. Roussel, 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 [3]. The same chromatographic procedures were used to purify γ2II14 and the γ2II14 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-
crucial for binding [11]. We therefore prepared T2III4 in recom-

of disulfide-bonded loop a (Fig. 2) which has been shown to be

like motif )'2III4, with a high sequence identity (77%) to the
laminins in the immobilized form (data not shown).

served in a direct nidogen binding assay [3, 24] using both

difference between laminin-5 and fragment P1 was also ob-

0.1nM. Human laminin-7 (ct3f1271) had a similar activity

has been attributed [5], with ICs0 values in the range 0.06-

its recombinant subfragment y lIII4, to which the entire activity

fragment P1 could be completely blocked by fragment P1 and

novel laminin isoforms (Fig. 1). The interaction of nidogen and

~lfllyl) was used to examine nidogen binding affinity of two

3. Results

A radioligand assay consisting of soluble recombinant nidogen and

125I-labelled laminin-1 fragment P1 was used in competition tests for the
determination of relative affinities based on ICs0 (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

50%) values [3, 9, 14]. Direct binding was analyzed in solid-phase assays

Inhibitors used were mouse fragment P1 (o) and its ylIII4 motif (e),
human laminin-5 (-) and laminin-7 (A), the y2III4 motif of human
laminin y2 chain (U) and mutant y2III4 (u) with Ser526 to Asn and Ser526
to Val substitutions.

2.3. Binding assays and immunological methods

A radioligand assay consisting of soluble recombinant nidogen and

125I-labelled laminin-1 fragment P1 was used in competition tests for the
determination of relative affinities based on ICs0 (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 y2III4 by subcutaneous injections with complete

Freund's adjuvant at intervals of 3-4 weeks. ELISA and immunoblot-
ting followed routine protocols.

3. Results

A radiological competition assay involving nidogen and 125I-

labelled fragment P1 from mouse laminin-1 (chain composition

a1b1g1) 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 y1III4, to which the entire activity

has been attributed [5], with ICs0 values in the range 0.06–

0.1nM. Human laminin-7 (a3b2g2y1) had a similar activity

(ICs0 = 0.15 nM) while laminin-5 (a3b3g2y2) had an activity too

low to be measured (ICs0 > 20 nM). A more than 100-fold difference

between laminin-5 and fragment P1 was also ob-

served in a direct nidogen binding assay [3, 24] using both

laminins in the immobilized form (data not shown).

Domain III of the y2 chain of laminin-5 possesses an EGF-

like motif y2III4, with a high sequence identity (77%) to the

nidogen-binding motif y1III4 of the y1 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 y2III4 in recom-

binant 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 y1III4 indicating a molecular mass of about 7 kDa (Fig. 3A).
With an ICs0 = 13 μM, however, its affinity for nidogen was 100,000-fold lower than y1III4 (Fig. 1). This

affinity was even distinctly lower than that of synthetic hep-
tapeptide NIDPNAV (ICs0 = 0.4 μM) designed according to a

y1III4 sequence [11]. We therefore prepared a y2III4 double

mutant where Ser526 and Ser528 within the heptapeptide binding

region were converted to Asn and Val, respectively, corre-

sponding to the substitutions found in y1III4 (Fig. 2). The

recombinant mutant showed a comparable electrophoretic mobi-

ity to y2III4 (Fig. 3B) but had an even higher nidogen affinity

(ICs0 = 0.4 nM), approaching that of y1III4 (Fig. 1). Thus a

very restricted change in the sequence is responsible for a con-

siderable improvement in binding.

It was also of interest to compare immunological epitopes of

y2III4 with those of y1III4. An antiserum raised against

y2III4, showed a strong reaction with y2III4 in ELISA but a

considerable improvement in binding.

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 lamin EGF-like motif γ2III4 (top) and comparison with the homologous nidogen binding motif γ1III4 (bottom) of mouse lamin. Only the substitutions are shown for γ1III4. The sequences correspond to positions 516–571 of the γ chain [21]; including signal peptide) and to positions 792–847 of the γ 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.

(Kd = 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.

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 γ2II1I4 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 γ2II1I4 back to the γ1 chain substitutions, producing a γ2II1I4 mutant almost as active as γ1III4. Other residues crucial for binding are conserved in the heptapeptide region of γ2II1I4 (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 in 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.

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