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Two non-contiguous regions contribute to nidogen binding to a single EGF-like motif of the laminin γ1 chain

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High affinity binding of nidogen to laminin is mediated by an EGF-like repeat γ1III4 of the mouse laminin γ1 chain and has now been restricted to two short non-contiguous regions of its 56 residue sequence by use of synthetic peptides and recombinant mutants. Disulfide loop a,b of the repeat and a modified loop a,c could completely inhibit binding, with a 5000-fold or 300-fold reduced affinity respectively. Synthetic loops c and d lacked inhibitory activity. Some binding contribution of Tyr819 in loop c was, however, shown by mutation and side chain modification. Together with studies of loop chimeras, this indicated a distinct cooperativity between the two binding sites. The major binding site of loop a was localized to the heptapeptide NIDPNAV (position 798–804). A change of Asp800 to Asn or Ala803 to Val caused a strong reduction in binding activity, while only small effects were observed for the changes Pro801 to Gln and Ile799 to Val. The latter replacement corresponds to the single substitution found in the same region of the *Drosophila* laminin γ1 chain. However, the changes Asn802 to Ser or Val804 to Ser, both known to exist in the laminin γ2 chain, were deleterious mutations. This demonstrated conservation of binding structures in laminins of distantly related species, but not between homologous chains of laminin isoforms.

Key words: basement membranes/inhibitory peptides/protein interaction/recombinant mutant

Introduction

Various isoforms of cross-shaped laminins have been identified as major cell-adhesive and structural proteins of basement membranes and other extracellular structures (Engel, 1993; Timpl and Brown, 1994). They are large multidomain proteins (600–900 kDa) and consist of disulfide-linked α, β and γ chains (for recent nomenclature see Burgeson *et al.*, 1994; Timpl and Brown, 1994). Many heterotypic interaction sites have been demonstrated for laminin 1, of chain composition α1β1γ1, including a single high affinity binding site (K_D = 0.5 nM) for the 150 kDa basement membrane protein nidogen (Fox *et al.*, 1991). Nidogen also binds to collagen IV, the proteoglycan perlecan and other extracellular ligands and thus mediates the formation of ternary complexes between laminin 1 and the other components (Brown *et al.*, 1994; Mayer and Timpl, 1994). Nidogen binding to laminins seems therefore to be a critical step in the supramolecular assembly of basement membranes. This interpretation was recently underscored in studies with antibodies which block the nidogen binding site of laminin 1 (Mayer *et al.*, 1993) and inhibit kidney tubulogenesis and lung branching in embryonic organ cultures (Ekblom *et al.*, 1994).

The high affinity nidogen binding site has been localized to a single motif homologous to epidermal growth factor (EGF) present in the short arm domain III of the mouse laminin γ1 chain (Gerl *et al.*, 1991; Mayer *et al.*, 1993). This laminin EGF-like repeat γ1III4 consists of 56 residues and four disulfide-linked loops (a–d), as indicated from the homology to EGF (Cooke *et al.*, 1987; Montelione *et al.*, 1987) and other representative sequence features (Sasaki and Yamada, 1987; Engel, 1989). The same affinity for nidogen binding was also observed with human laminins 2 and 4, of chain compositions α2β1γ1 and α2β2γ1 (Brown *et al.*, 1994), and is explained by the 97% sequence identity of the mouse and human γ1III4 structure (Pikkarainen *et al.*, 1987). A lower sequence identity (61%) has been shown for this repeat in *Drosophila* laminin γ1 chain (Chi and Hui, 1989) and the human laminin γ2 chain isoform (77%; Kallunki *et al.*, 1992). This has raised the intriguing question of whether these laminins also have affinity for nidogen and makes the more precise mapping of binding structures in the mouse repeat γ1III4 necessary for molecular interpretations.

Despite the huge number of EGF-like repeats identified in many extracellular and membrane-bound proteins (see Rees *et al.*, 1988; Selander-Sunnerhagen *et al.*, 1992), little is known about their binding properties and the structures involved. Biological evidence for the importance of these repeats comes from studies of fibrillin mutants which are considered to cause Marfan syndrome (Dietz *et al.*, 1991; Lee *et al.*, 1991) and from lethal mutations in the neurogenic *Drosophila* protein notch (Kelley *et al.*, 1987), but the functional basis is not yet understood. A precise identification has, however, been achieved for calcium binding sequences present in loop a and interloop regions of EGF-like repeats from several coagulation factors (Handford *et al.*, 1991; Selander-Sunnerhagen *et al.*, 1992). Site-directed mutagenesis was also used to map the receptor binding site of EGF and demonstrated crucial residues at the C-terminal end beyond loop c and in the hinge region between loops b and c (Moy *et al.*, 1989; Campion *et al.*, 1992, 1993).

In view of the potential importance of nidogen binding

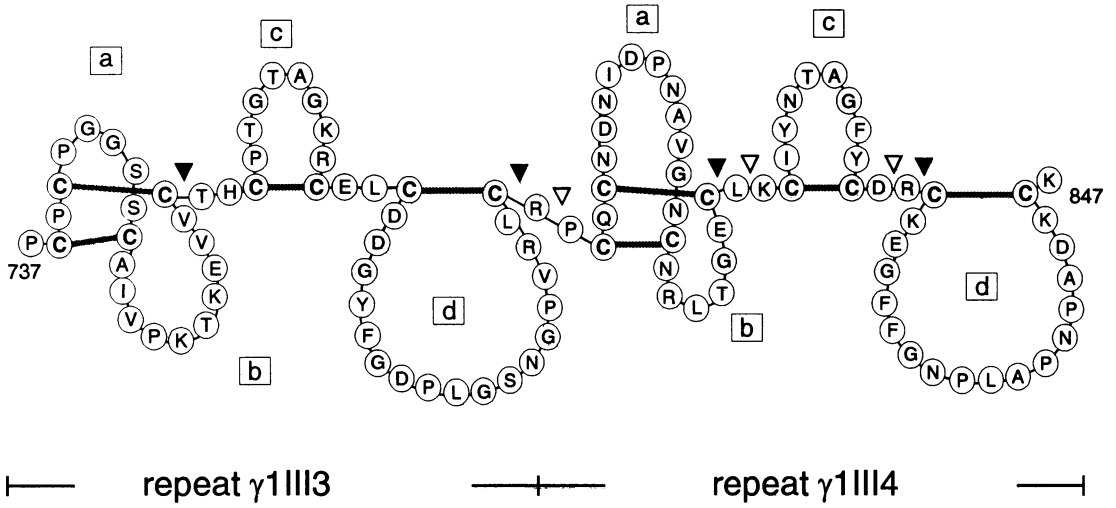

Fig. 1. Amino acid sequence of EGF-like repeats 3 and 4 in domain III of laminin γ1 chain. The disulfide bonds were predicted from those of EGF (loops a, b and c) and an extra cysteine pair involved in loop d (Engel, 1989). Arrowheads mark the borders of synthetic peptides (▽) or of recombinant deletion mutants and chimeras (▼).

to laminin we have now undertaken the task of a more precise mapping of binding structures in the EGF-like repeat γ1III4 by a combination of recombinant and synthetic approaches. This allowed the mapping of individual residues essential for binding to restricted non-contiguous regions within two disulfide loops. Some insights were also obtained into the importance of species and isoform variations which could be relevant in a biological context.

Results

The nidogen binding EGF-like repeat 4 of laminin γ1 chain domain III (γIII4) consists of 56 amino acid residues which are folded into four loops (a–d) by disulfide bonds (Figure 1). This sequence is unique within the γ1 chain (Engel, 1993), which explains the high specificity found for nidogen binding (Gerl *et al.*, 1991; Mayer *et al.*, 1993). It is compared in Figure 1 with the repeat 3 sequence which was used for chimeric constructs. Since it is likely that not all regions of repeat γ1III4 are equally important for affinity, a more precise mapping of binding sites has now been accomplished with fragments or mutants which were prepared by peptide synthesis or recombinant methods. Their binding activities were determined in a sensitive radioligand competition assay using authentic laminin fragment P1 or related recombinant fragments as reference inhibitors (Figure 2).

Effects of side chain modifications

Previous circumstantial evidence from radioligand assays has indicated that one of the two tyrosines in loop c of repeat γ1III4 contributes to binding (Mayer *et al.*, 1993). In order to confirm and extend such observations, which could be valuable for the design of mutants, we resorted to several efficient side chain modification procedures. This was done with recombinant fragment γ1III3–5, consisting of repeats 3, 4 and 5, which was available in large quantities. Modification of tyrosine by two different methods caused a 75-fold decrease in binding activity (Table I). A similar decrease was observed after modification of Asp and Glu, while a lower effect (7-fold) was obtained after acetylation of Lys.

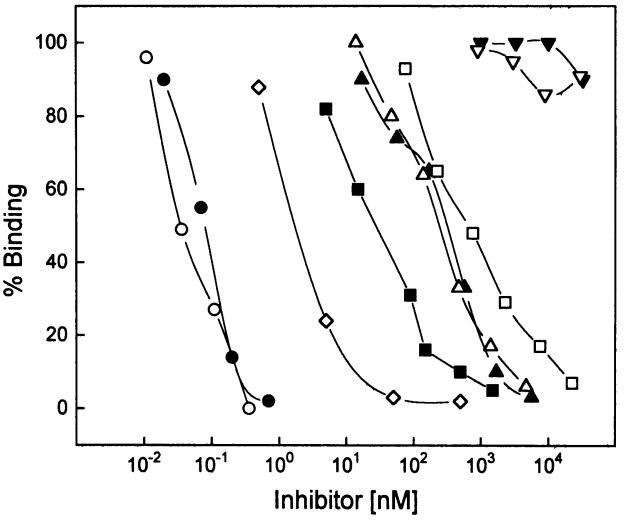

Fig. 2. Inhibition of radioligand binding between nidogen and laminin fragment P1 by synthetic γ1III peptides and modified fragments. The inhibitors used were laminin fragment P1 (●), recombinant fragment γ1III3–5 (○), nitrated γ1III3–5 (◇) and synthetic oxidized loop a,b (▲), non-oxidized loop a,b (△), oxidized loop c (▼), oxidized loop d (▽), oxidized loop a,c (■) and synthetic NIDPNAV (□).

Binding activity of synthetic disulfide loop structures and of smaller peptides

Three large fragments corresponding to different disulfide loops of repeat γ1III4 were obtained by peptide synthesis (Figure 1) and the two (loop a,b) or single (loop c and loop d) disulfide bonds of these products were correctly connected by oxidation. Proper disulfide bond formation and deprotection of the purified peptides was assured by mass spectrometry. Competition assays with loop a,b in the oxidized and the non-oxidized forms showed that both could completely inhibit laminin–nidogen binding in an equal fashion. Their IC_{50} values (400 nM) were, however, 5000-fold higher than that obtained with fragment P1

Table I. Effects of side chain modification of recombinant γ1III3–5 on the inhibitory capacity for laminin–nidogen binding

Modification	IC_{50} (nM)
None	0.04
Tyr, nitration	3
Tyr, acetylation	3
Asp/Glu, amidation	2.1
Lys, acetylation	0.27

(Table II). No inhibitory activity was observed for the oxidized loop c and loop d peptides (IC_{50} > 90–200 μM). Amidation of loop a,b caused a distinct decrease in activity, in agreement with similar observations for a larger and more active fragment (Table I).

A modified peptide corresponding to loop a,c was also synthesized by deleting the first and third cysteines and seven additional residues from loop b. The first and second pairs of remaining cysteines were then oxidized, yielding the structure QCNDNIDPNAVGCLKCIYNTAGFYCD. This peptide had a 20-fold higher inhibitory activity (IC_{50} = 22 nM) compared with loop a,b and was only 300-fold less active than fragment P1 (Table II). This indicates cooperation between binding sites present in loops a and c for high affinity association to nidogen, as already suggested from the tyrosine modification experiments. Cooperation between loops a and c needs a covalent connection, since the activity of loop a,b did not increase upon addition of an equimolar amount of loop c peptide (Table II).

The non-oxidized loop a,b peptide (24 residues) was used in proteolysis experiments to define its binding site (Table II). Cleavage at arginine by trypsin yielded the C-terminal hexapeptide T2, with low activity, and a larger T1 peptide, with undiminished activity. Thermolysin digestion of T1 released an inactive C-terminal peptide Th1, demonstrating that loop a is responsible for binding. Cleavage at Asp residues with endoproteinase Asp-N released an inactive N-terminal pentapeptide E3 and a 19 residue peptide E1, with almost unchanged activity (IC_{50} = 530 nM). A further cleavage product E2, which lacked the N-terminal sequence Asp–Asn–Ile of E1, showed, however, a significant loss in activity (IC_{50} = 3 μM).

Together these data suggested that NIDPNAVGN could be a minimal binding sequence, which was confirmed with a synthetic peptide showing only a marginal loss in activity (IC_{50} = 600 nM) when compared with loop a,b (Table II). A further synthetic heptapeptide NIDPNAV was almost as active (IC_{50} = 800 nM). Removal of the N-terminal Asn produced, however, a 2-fold loss in activity (IC_{50} = 1.6 μM). The inhibitory activity was abolished by a larger N-terminal deletion (NID), as well as by removal of the C-terminal Val. This showed that the entire heptapeptide NIDPNAV (position 798–804 in the γ1 chain) represents the binding site of loop a.

Studies with synthetic variants of the loop a heptapeptide

The contribution of single residues to the inhibitory activity of the heptapeptide was further studied by synthetic homologs containing one to three replacements (Table III). The change of D800 to N produced a 100-fold reduction in activity and is consistent with a comparable loss observed after modification of carboxyl groups (Tables I and II). Surprisingly, the change of P801 to Q caused only a 3-fold decrease. A moderate 16-fold decrease was observed for the conservative A803 to V substitution.

Further replacements were designed from comparison with the homologous laminin sequences. The *Drosophila* γ1 chain (Chi and Hui, 1989) shows only a single conservative I799 to V substitution in that heptapeptide region. This substitution, when examined as a synthetic peptide, had only a marginal effect on affinity (Table III). The human laminin γ2 chain isoform (Kallunki *et al.*, 1992) shows, in addition to the change found for *Drosophila* laminin, an N802 to S and V804 to S change. The additional introduction into the heptapeptide sequence of each replacement, separately, or both together, in each case caused inactivation (Table III).

Production and activity of chimeric protein motifs and site-directed mutants

Our previous studies have shown that it is feasible to obtain individual or tandem arrangements of EGF-like repeats of laminin γ1 chain in sufficient quantities from

Table II. Inhibitory capacity of synthetic loops of EGF-like repeat 4 and of smaller synthetic and proteolytic fragments

Origin position	Sequence or equivalent description	IC_{50} (nM)
Laminin	fragment P1	0.08
S, 792–815	loop a,b, oxidized	380
S, 792–815	loop a,b, non–oxidized	400
S, 792–815	loop a,b, amidated	> 5400
S, 816–827	loop c, oxidized	> 200 000
S, 828–847	loop d, oxidized	> 90 000
S, 794–805/815–827	loop a,c, oxidized	22
S, mixture	loop a,b + loop c	420
T1, 792–809	PCQCNDNIDPNAVGNCNR	500
T2, 810–815	LTGECL	12 000
Th1, 803–809	AVGNCNR	> 30.000
E1, 797–815	DNIDPNAVGNCNRLTGECL	530
E2, 800–815	DPNAVGNCNRLTGECL	3000
E3, 792–796	PCQCN	> 96 000
S, 798–806	NIDPNAVGN	600
S, 798–804	NIDPNAV	800
S, 799–806	IDPNAVGN	1600
S, 798–803	NIDPNA	> 460 000

All peptides were synthetic (S) and several obtained by further digestion with trypsin (T), thermolysin (Th) and endoproteinase Asp-N (E). For position numbers and loop identification see Figure 1.

Table III. Effects of amino acid replacements in the heptapeptide NIDPNAV on inhibitory capacity for laminin–nidogen binding

Peptide	IC_{50} (nM)
NIDPNAV	900
NI**N**PNAV	100 000
NID**Q**NAV	2400
NIDPN**V**V	16 000
N**V**DPNAV	1200
N**V**DP**S**AV	> 180 000
N**V**DPNA**S**	> 160 000
N**V**DP**S**A**S**	> 290 000

Substitutions are shown in bold letters.

transfected human cell clones as long as the repeats were connected to a signal peptide sequence (Mayer *et al.*, 1993). The application of the same approach to deletion mutants of the nidogen binding EGF-like repeat lacking either loop d or loop a,b (Figure 1), however, did not show a corresponding protein product in the culture medium. For several loop d deletion clones we could demonstrate the presence of substantial amounts of specific mRNA by Northern hybridization, suggesting intracellular degradation of their protein product. This indicates that the integrity of the four loop structure is essential for proper folding and secretion.

Because of this failure, we changed to the strategy of producing chimeras by exchanging loop a,b and loop c,d mutually between the inactive repeat 3 and the active repeat 4 and had no difficulties in obtaining the corresponding fragments γ1III 3ab4cd and γ1III 4ab3cd. Further mutants were generated by site-directed mutagenesis and included one or both tyrosines in loop c (fragments γ1III4Y819-A, γ1III4Y825-A and γ1III4Y819/825-A) or residues in loop a (fragments γ1III4D800-N and γ1IIIN802-S), as suggested from the heptapeptide experiments. In addition, we prepared the deletion of a whole EGF-like repeat by joining repeats 3 and 5 (fragment γ1III3-5Δ4). With all these expression constructs the corresponding protein products could be obtained and purified and showed in SDS–gel electrophoresis a single band similar to those described for other recombinant EGF-like repeats (Mayer *et al.*, 1993). They were all disulfide bonded, as indicated by a decrease in electrophoretic mobility when examined in non-reduced form and by their resistance to pepsin (data not shown).

Inhibition studies with the chimeric fragments (Table IV) demonstrated a distinct decrease in activity for fragment γ1III4ab3cd (IC_{50} = 80 nM) compared with the wild-type γ1III4 (0.05 nM) and its activity was only 3-fold higher compared with synthetic oxidized loop a,b. An even stronger decrease was noticed for chimeric fragment γ1III3ab4cd, underscoring previous observations on the dual role of loops a and c in binding. The activities of the chimeras are due to the EGF-like repeat 4, since the deletion mutant γ1III3-5Δ4 was without measurable activity.

Table IV. Inhibitory activity of recombinant mutants and chimeric structures of the EGF-like repeat γ1III4 for laminin–nidogen binding

Inhibitor	IC_{50} (nM)
Laminin fragment P1	0.05
γIII4	0.05
loop a,b oxidized	270
γ1III4ab3cd	80
γ1III4ab3cd, reduced	520
γ1III3ab4cd	1100
γ1III3ab4cd, reduced	6700
γ1III4Y819-A	3
γ1III4Y825-A	0.06
γ1III4Y819/825-A	1.5
γ1III4Y819/825-A, reduced	430
γ1III4D800-N	1000
γ1III4N802-S	5500
γ1III3-5Δ4	> 3200

From the three tyrosine mutants studied, only those including Y819 showed a 30- to 60-fold reduction in activity (Table IV). The loss was of the same magnitude as that observed after chemical modification (Table I). The single residue mutation γ1III4Y825-A was, however, without significant effect on inhibitory activity. A dramatic decrease in activity (~100 000-fold) was observed with the mutant γ1III4N802-S, affecting the heptapeptide binding region. Interestingly, this fragment was even 5-fold less active than the chimera γ1III3ab4cd, where the entire loop a,b has been replaced by another, smaller loop structure (see Figure 1). A further mutation within the heptapeptide region (fragment γ1III4D800-N) showed a similar decrease in activity compared with fragment γ1III3ab4cd (Table IV). These changes correspond to those observed with synthetic variants of the heptapeptide (Table III).

A few selected mutants were reduced and alkylated under denaturing conditions in order to examine the contribution of disulfide bonds to binding activity (Table IV). The largest relative decrease in activity (~300-fold) down to the level of the loop a,b peptide was found for γ1III4Y819/825-A. Reduced γ1III4ab3cd had about the same activity, indicating that loop a,b, when present in a whole EGF-like motif, is to some extent more active in the disulfide-bonded form. Reduction of γ1III3ab4cd caused a further 6-fold decrease in its already rather low activity. The data therefore show some small disulfide dependence of the loops a and c binding regions when examined separately with appropriate recombinant proteins. The high affinity cooperation between both sites seems to be much more dependent, as demonstrated with γ1III4Y819/825-A.

Discussion

Nidogen binding to the laminin γ1 chain is apparently a crucial step in basement membrane assembly, as indicated from binding studies and certain developmental models (Ekblom *et al.*, 1994; Mayer and Timpl, 1994). The binding structure has been localized to a single EGF-like motif γ1III4 of laminin (Mayer *et al.*, 1993). This motif was small enough to approach a more precise identification of binding sites by a combination of recombinant and chemical methods . The data demonstrated that disulfide loops a and c of γ1III4 are indispensable for high affinity binding. Each segment alone had either a 5000-fold reduced affinity (loop a) or an affinity which was too low to measure (loop c). A similar difference was also shown with chimeric constructs, indicating a stronger contribution to binding for loop a than loop c. Furthermore, the connection of loops a and c by an artificial peptide link increased the affinity by a factor of 20. This emphasizes that a close spatial relationship between both loops is required for the expression of high binding activity.

The low binding activity of loop a could be mapped to the heptapeptide sequence NIDPNAV (Figure 3). A search of the protein data bank demonstrated this sequence only in mouse and human laminin γ1 chains. Several sequences with one or two substitutions could be detected in unrelated proteins, but always included a change in one of the four residues which are critical for binding. Synthetic variations of the heptapeptide, as well as chemical modifications, showed a strong contribution to binding of the β-carboxyl

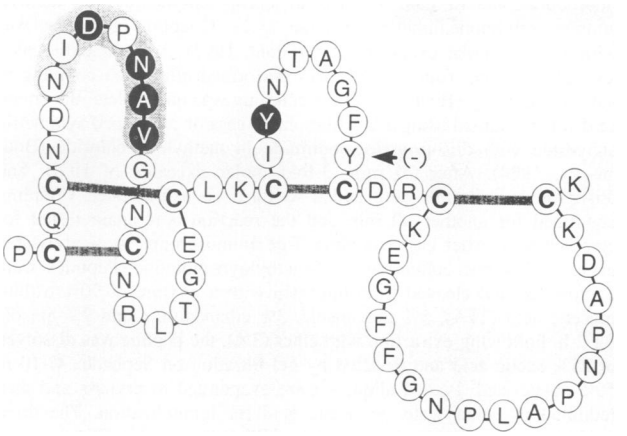

Fig. 3. Map of the nidogen binding site in the EGF-like repeat γ1III4 of laminin. The relative contribution of individual residues is indicated within the circles and correlate to a <10-fold (white circle), a 10- to 100-fold (shaded) and >100-fold (black) loss of affinity if the residues are changed as described in the text. (−) indicates no contribution as judged from site-directed mutagenesis of γ1III4.

group of Asp. The central Asn and C-terminal Val when changed to Ser also caused a considerable loss of activity. The more conservative replacement of Ala by Val still decreased activity by a factor of 16. This indicates that these four residues could provide essential contact sites for nidogen binding. The other three residues, including the N-terminal Asn−Ile and the central Pro, seem to be of much less importance, as shown either by fragmentation or by substitutions. A pentapeptide DPNAV may therefore still have significant binding activity, but this was not shown directly. The low contribution of Pro to binding was surprising in view of the abundant evidence that Pro is essential for β turns in loop structures. Yet the binding activity of synthetic loop a was not dependent on disulfide bonds, suggesting that the loop may have a more flexible conformation.

Further evidence that loop c is also important for high affinity binding, despite its lack of activity when analyzed as a synthetic product, was derived from chemical modification and recombinant studies. These showed that Tyr819, but not Tyr825, contributes to binding and its modification caused an ~60-fold reduction in affinity. Replacement of γ1III4 loop c by another loop c from a non-binding EGF-like repeat (fragment γ1III4ab3cd; see Table IV) produced a further 30-fold decrease in activity. This indicates involvement in high affinity binding of other loop c residues, besides Tyr819, which remain to be identified. Our fragmentation data also indicate that loop b is not essential for binding. However, we have no information on the importance of its connecting Leu−Lys link to loop c. A similar link in EGF was found to be important for receptor binding (Campion *et al.*, 1993). Oxidized loop d was also an inactive inhibitor, but, in view of the same data for oxidized loop c, it may require further site-directed mutagenesis data for final conclusions to be drawn. Such a role seems unlikely, since, as discussed below, loop d of mouse and *Drosophila* γ1 chains shows only 31% sequence identity (Chi and Hui, 1989) but both laminins bind strongly to mouse nidogen. However, EGF and many other homologous motifs lack a loop d structure or possess a larger C-terminal sequence not linked by a disulfide bridge. This sequence was shown to be essential for the function of EGF (Moy *et al.*, 1989; Campion *et al.*, 1992).

Loop a of the mouse and *Drosophila* γ1III4 repeat shows a much higher sequence identity (88%), but includes an Ile to Val substitution in the heptapeptide binding region. Studies with synthetic peptide variants, however, did not reveal any significant change in binding activity. The essential Tyr819 of loop c is replaced by His in *Drosophila* (Chi and Hui, 1989). Preliminary radioligand assay data (L.Fessler and R.Timpl, unpublished results), however, showed a comparable inhibitor activity of mouse and *Drosophila* laminin 1 for binding to mouse nidogen. This indicates a neutral mutation and emphasizes again that other residues of loop c, which are conserved in both laminins, may be more important for high affinity binding.

The γ1 chain, which is found in several laminins (Timpl and Brown, 1994), is replaced by the homologous γ2 chain in laminin 5, previously referred to as kalinin/nicein (Rousselle *et al.*, 1991; Marinkovich *et al.*, 1993). Laminin 5 has a restricted distribution in epidermal anchoring filaments and there may serve special functions (Burgeson, 1993). This laminin has the same Ile to Val replacement in the loop a heptapeptide sequence as *Drosophila* laminin, but in addition two Ser replace the central Asn and the Val (Kallunki *et al.*, 1992). Both of the latter replacements inactivate binding, as shown by synthetic variants and recombinant mutants. This predicts that laminin 5 has only a low affinity, if any, for nidogen. Together these data point to the interspecies conservation of binding structures within laminin 1 and some other isoforms, but with modulation of binding activities depending on the γ chain variant. This functional variability could also include different α and β chain isoforms, for which some evidence has been reported (Brown *et al.*, 1994).

Our data strongly indicate two restricted contact regions for nidogen binding in the laminin γ1 chain. We cannot exclude the possibility that the less active loop c stabilizes the conformation of loop a, rather than being directly involved in binding, but this appears less likely. Actually, the product of the low affinities (10^{-13} M) of the two loop chimeras γ1III4ab3cd and γ1III3ab4cd is only two orders of magnitude smaller than the highest inhibiting activity observed for the wild-type product (Mayer *et al.*, 1993). This indicates cooperativity between two binding sites located in loops a and c, even though their binding energies are not fully additive. Further interpretations will depend on three-dimensional structural analyses of the binding sites. Crystals of the fragment γ1III3–5 with reasonable X-ray diffraction patterns have been obtained and may be suitable for this approach (J.Stetefeld, R.Timpl and R.Huber, unpublished results). The binding fragment γ1III4 is also small enough to allow a straightforward NMR analysis. Preliminary data with the synthetic binding loop a nonapeptide (Table II) have indicated a mainly random coil conformation (T.Holak and R.Timpl, unpublished results). These studies are in progress and will hopefully provide a precise conformational picture of the binding sites.

Materials and methods

Expression of recombinant EGF-like repeats

The vectors used to obtain individual or in-tandem arranged repeats γ1III3–5, γ1III3–4, γ1III3 and γ1III4 (γ1III replaces the previously used

term B2III; see Burgeson *et al.*, 1994) have been described (Mayer *et al.*, 1993). These vectors and mutated vectors (see below) were used to transfect human embryonic kidney cells 293, and stable transfectants were selected by puromycin or G418 treatment (Nischt *et al.*, 1991). Stably transfected clones were characterized by Northern hybridization for mRNA expression and by SDS–PAGE of serum-free culture medium to identify clones which efficiently produce and secrete the processed protein (Nischt *et al.*, 1991). Purification of the recombinant fragments by DEAE–cellulose and molecular sieve chromatography followed previously used protocols (Mayer *et al.*, 1993).

Construction of expression vectors for γ1III4 mutants

We followed a previous strategy where the desired fragment was fused via a *Nhe*I site to the signal peptide of human BM-40 to allow processing and secretion of the recombinant product (Mayer *et al.*, 1993). Mutations, additional restriction sites and stop codons were introduced by primers using PCR amplification with Vent polymerase (Biolabs) following the supplier's instructions. The primers K24, K25, K27 and Ti1 have been described in Mayer *et al.* (1993) and the following additional oligonucleotides were used in this study (mutated sequences are underlined):

3ab4S: 5′- AAGGAAGTGGTGTGCCTGAAGTGCATC
3ab4R: 5′- GATGCACTTCAGGCACACCACTTCCTT
4ab3S: 5′- GACGGGCGAGTGCACGCACTGTCC
4ab3R: 5′- GGACAGTGCGTGCACTCGCCCGTC
SYwt: 5′- AACACGGCTGGTTTCTAC
SYmut: 5′- AACACGGCTGGTTTCGCCTGCGACCGGTGC
RYwt: 5′- GAAACCAGCCGTGTTATA
RYmut: 5′- GAAACCAGCCGTGTTAGCGATGCACTTCAGGC
ΔR: 5′- GCGCAGGCTTTGCACAGTCTCACGG
ΔS: 5′- CCGTGAGACTGTGCAAAGCCTGCGC
N-Mut/R: 5′- CAACCGCGCTGGGGTCTATGTTGTC
Mut-N: 5′- ACATAGACCCCAGCGCGGTTGGCAAC
Mut-D: 5′- AACGACAACATAAACCCCAACGCGGT
Mut-Xba: 5′- CGAGCATGCATCATGAGGGCCCTATT.

Construct γ1III3–5Δ4 was generated by amplification of subfragments from the γ1III3–5 vector with primers Ti1×ΔR or ΔS×K24, fusion of the fragments and amplification with terminal primers Ti1×K24. Construct γ1III3ab4cd was generated by amplification of fragments from γ1III3–4 with primers Ti1×3ab4R and 3ab4S×K27, fusion of fragments and amplification with Ti1×K27. Construct γ1III4ab3cd was generated by amplification of fragments from γ1III4 with primers Ti1×4ab3R and from γ1III3 with primers 4ab3S×K25, fusion and amplification with Ti1×K25. All fragments were restricted with *Xba*I and *Xho*I and cloned into the corresponding sites of expression vector pCis (Gorman *et al.*, 1990).

Mutations of Tyr819 (TAT) and Tyr825 (TAC) to Ala (GCT or GCC) were introduced by PCR amplification of fragments by the use of primers Ti1×RYmut (resulting in product E1), Ti1×RYwt (E2), SYmut×K27 (E3) and SYwt×K27 (E4). Fragments E1 and E2 were fused in construct γ1III4Y819-A, E2 and E3 were fused in γ1III4Y825-A and both mutations were combined by fusion of E1 and E3 to yield construct γ1III4Y819/825-A. All fused fragments were amplified with primers Ti1×K27, restricted with *Xba*I and *Xho*I and cloned into pCis.

The construct pRC/D4 was generated by insertion of the *Hin*dIII–*Not*I insert of γ1III4 into the vector pRC/CMV (Invitrogen) and was used alternatively for the expression of this subdomain. Construct γ1III4D800-N contains a mutation of Asp800 (GAC) to Asn (AAC), which was introduced into pRC/D4 according to the transformer mutagenesis system (Clontech). Mutations were introduced by oligonucleotides Mut-D and Mut-Xba and selection for mutated plasmids was by digestion with *Xba*I as described by the supplier. Correct clones were selected after sequencing of the correponding region. Construct γ1III4N802-S, containing a mutation of Asn802 (AAC) to Ser (AGC), was generated from pRC/D4 by amplification with T7 (Biolabs)×N-Mut/R and Mut-N×K27. Fragments were fused, amplified by T7×K27, purified, restricted with *Nhe*I and *Xho*I and inserted into pCis-derived γ1III4, cleaved with *Nhe*I and *Xho*I. All constructs were verified by DNA sequencing of the whole insert.

Synthesis and characterization of peptides

Synthesis of peptides was carried out on polystyrene solid support using a Biosearch 9600 automated peptide synthesizer system. Amino acids protected with 9-fluorenylmethyloxycarbonyl were pre-activated as *N*-hydroxybenzotriazole (HOBt) esters that also contained an equimolar amount of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate and 1.5 equivalents of diisopropylethylamine in *N*-methylaminepyrrolidinone:dimethylsulfoxide (3:1). Coupling generally was with a 3-fold molar excess for 2 h (Grant, 1992). A qualitative Kaiser test (Stewart and Young, 1984) was performed after each coupling to monitor coupling efficiency. Where coupling was incomplete, the amino acid was recoupled using a 6-fold molar excess of preformed symmetric anhydride with diisopropylcarbodiimide in methylene chloride (Bodanszky, 1984). After 20 min, 3-fold molar excesses of HOBt and diisopropylethylamine were added to form an active ester. Coupling continued for another 90 min and the reaction was again tested for completeness. After each coupling, free amino groups were acetylated using 10% acetic anhydride in *N*-methylpyrrolidinone. Peptides were deprotected and cleaved from the resin with a mixture of 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol and 2% anisole for 2 h. Following extraction with ether (3×), the peptide was dissolved in 25% acetic acid and desalted by gel filtration on Sephadex G-10 in 50% acetic acid. Pooled aliquots were evaporated to dryness and then rediluted in either water or acetic acid for lyophilization. The dried product was purified by reverse phase HPLC using 0.1% TFA in water and acetonitrile gradients on a C18 column.

Peptides requiring oxidation of a single disulfide were dissolved in aqueous acetic acid to a concentration of <0.1 mg/ml. The pH was adjusted to 8.5 with ammonium hydroxide. The solution was loosely covered, bubbled with air and stirred until oxidation was complete. Completeness of oxidation was determined by Ellman's assay (Stewart and Young, 1984) and HPLC. The oxidized material was concentrated, desalted by gel filtration and lyophilized. Peptides requiring a second disulfide were synthesized with the second pair of cysteines protected by an acetamidomethyl (ACM) group. The first oxidation was performed as above. Following lyophilization of the singularly oxidized peptide, the ACM was removed by dissolving the peptide (0.05 nmol) in 350 ml methanol:water (1:6). The solution was maintained at room temperature and stirred while adding 50 ml 1 mM iodine in methanol dropwise over 1 h. The solution was cooled and concentrated to remove the methanol. The remaining solution was extracted with chloroform to remove the iodine. An Ellman's assay was performed to ensure completeness. Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry was performed to determine the correct product. Purity was assessed by reversed phase HPLC in several buffer systems.

Chemical and proteolytic modifications

Complete reduction of disulfide bonds was performed in 6 M guanidine–HCl, 0.05 M phosphate buffer, pH 8.0, with 0.02 M dithiothreitol (4 h, 37°C) followed by blocking with 0.08 M *N*-ethylmaleimide for 2 h at room temperature. Nitration of tyrosine was accomplished in 0.05 M Tris–HCl, pH 8.0, by adding a 2-fold molar excess of tetranitromethane for 1 h at room temperature (Riordan and Vallee, 1972). Selective acetylation of tyrosine hydroxyl groups was done in 1 M sodium acetate, pH 5.8, by adding a large excess of acetic acid anhydride for 30 min at room temperature (Ohnishi *et al.*, 1974). Selective acetylation of lysine amino groups was achieved in half-saturated sodium acetate solution by adding 5×2 μl acetic acid anhydride to 40 mg protein/80 ml incubated in an ice bath for 1 h (Fraenkel-Conrat *et al.*, 1957). Carboxyl groups (Asp, Glu) were activated with 1-ethyl-3-dimethylaminopropylcarbodiimide and then blocked by norleucine methylester (Hoare and Koshland, 1967). For proteolytic cleavage (24 h, 37°C) in 0.02 M NH_4HCO_3, trypsin (Worthington) and thermolysin (Merck) were used at a substrate:enzyme ratio of 1:25 and endoproteinase Asp-N (sequencing grade, Boehringer) at a ratio of 1:200. Proteolytic fragments and modified proteins were then purified by reverse phase HPLC and identified by amino acid analysis (Mayer *et al.*, 1991).

Binding assay

A radioligand competition assay was used for the measurement of relative binding affinities (Mann *et al.*, 1988; Fox *et al.*, 1991). A fixed concentration of recombinant nidogen (0.2 nM) was incubated overnight at 4°C with inhibitors at varying concentrations, followed by the addition of ^{125}I-labeled laminin fragment P1 (0.01 nM; 10 000–20 000 c.p.m.) for the same incubation period. Bound and non-bound fragment P1 was then separated by antibodies against nidogen. Competitor concentrations causing 50% inhibition (IC_{50}) were determined from dose–response profiles. All assays were calibrated with non-labeled fragment P1, which showed in 14 separate assays an average IC_{50} value (± SD) of 0.058 ± 0.026 nM. IC_{50} values compared from different assays were normalized to a single fragment P1 value.

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