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A single EGF-like motif of laminin is responsible for high affinity nidogen binding

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A major nidogen binding site of mouse laminin was previously localized to about three EGF-like repeats (Nos 3-5) of its B2 chain domain III [M.Gerl *et al.* (1991) *Eur. J. Biochem.*, 202, 167]. The corresponding cDNA was amplified by polymerase chain reaction and inserted into a eukaryotic expression vector tagged with a signal peptide. Stably transfected human kidney cell clones were shown to process and secrete the resulting fragment B2III3-5 in substantial quantities. It possessed high binding activity for recombinant nidogen in ligand assays, with an affinity comparable with that of authentic laminin fragments. In addition, complexes of B2III3-5 and nidogen could be efficiently converted into a covalent complex by cross-linking reagents. Proteolytic degradation of the covalent complex demonstrated the association of B2III3-5 with a ~80 residue segment of nidogen domain G3 to which laminin binding has previously been attributed. The correct formation of most of the 12 disulfide bridges in B2III3-5 was indicated from its protease resistance and the complete loss of cross-reacting epitopes as well as of nidogen-binding activity after reduction and alkylation. Smaller fragments were prepared by the same recombinant procedure and showed that combinations of EGF-like repeats 3-4 and 4-5 and the single repeat 4 but not repeats 3 or 5 possess full nidogen-binding activity. This identifies repeat 4 as the only binding structure. The sequence of repeat 4 is well conserved in the human and in part in the *Drosophila* laminin B2 chain. It was further shown that antibodies against B2III3-5 inhibit laminin binding to nidogen, indicating that repeat 4 represents the only high affinity binding site of laminin.

Key words: antibody inhibition/basement membranes/ protein interaction/recombinant expression

Introduction

Laminin is a major structural and cell-adhesive protein of basement membranes and is known to exist in tissues in various isoforms. The most extensively characterized isoform from the mouse Engelbreth-Holm-Swarm (EHS) tumor consists of three individual chains (B1, B2, A) which are connected to each other to form a cross-shaped structure of about 850 kDa (Beck *et al.*, 1990; Paulsson, 1992; Engel, 1992). Laminin contributes to the supramolecular organization of basement membranes by self assembly into large networks (Yurchenco *et al.*, 1992) and by binding to collagen IV, the proteoglycan perlecan (Laurie *et al.*, 1986; Aumailley *et al.*, 1989; Battaglia *et al.*, 1992) and the 150 kDa protein nidogen (Paulsson *et al.*, 1987; Mann *et al.*, 1988). The latter binding seems to be of particular functional importance since this allows the association of the laminin-nidogen complex with either collagen IV or perlecan (Fox *et al.*, 1991; Battaglia *et al.*, 1992). The structure of nidogen is characterized by three globular domains, G1-G3, that are separated from each other by either a stiff rod (G2, G3) or a flexible segment (G1, G2). This molecular architecture separates the laminin-binding domain G3 from the collagen/proteoglycan-binding domain G2 by a distance of ~15 nm and thus permits multiple interactions (Fox *et al.*, 1991; Reinhardt, 1992). These observations also suggest that nidogen plays a key role in the assembly of basement membranes (Aumailley *et al.*, 1993).

Electron microscopy of the laminin-nidogen complex has shown that only a single nidogen molecule binds to one of the short arms of laminin (Paulsson *et al.*, 1987). Further biochemical studies assigned nidogen binding activity exclusively to laminin fragment P1 (Mann *et al.*, 1988). Fragment P1 corresponds to the inner rod-like segments of the three short arms of laminin and consists of 29 EGF-like repeats contributed by all three constituent chains (Sasaki *et al.*, 1988; Beck *et al.*, 1990; Engel, 1993). These 50-60 residue repeats contain eight cysteines in disulfide-bonded form and it was shown that intact disulfide bridges are essential for nidogen binding (Mann *et al.*, 1988). By a combination of partial reduction and further proteolysis it was, however, possible to localize the binding activity of fragment P1 to a 28 kDa fragment (Gerl *et al.*, 1991). The small fragment originated from laminin B2 chain domain III (Sasaki and Yamada, 1987) and consisted of the EGF-like repeat Nos 4 and 5 and portions of repeat Nos 3 and 6. Further degradation of this structure resulted in inactivation of nidogen binding (Gerl *et al.*, 1991).

Because of the inherent difficulties in obtaining the 28 kDa fragment in substantial quantities and in mapping its binding activity to smaller structures, we have now resorted to a recombinant approach based on eukaryotic expression vectors and transfected human cell clones. This approach has already successfully been used for nidogen and several other extracellular matrix proteins or their fragments (Fox *et al.*, 1991; Nischt *et al.*, 1991; Specks *et al.*, 1992). Since the designed fragments to be expressed are located in a central position in laminin, we developed a new strategy based on a versatile expression vector that joins them to a signal peptide in order to assure the proper processing and

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secretion of the protein products to the culture medium. This allowed the recombinant production of a highly active fragment consisting of repeats 3–5 and assignment of its nidogen-binding activity to a single EGF-like structure.

Results

Design of expression vectors and transfection of cells

Nidogen binding of laminin has been attributed to three EGF-like repeats from a central region of its B2 chain (Gerl *et al.*, 1991). Each repeat has a unique sequence with almost only the eight cysteine residues being strictly conserved (Figure 1). Expression plasmids for the recombinant production of this structure (fragment B2III3–5) as well as of individual repeats and combinations of two of them (Table I) were prepared by amplification of appropriate regions from cDNA clones (Sasaki and Yamada, 1987) by polymerase chain reaction (PCR). Authentic oligonucleotide primers used for the 5′-ends introduced a single additional *Nhe*I restriction site. Primers for the 3′-ends contained besides the authentic laminin sequence a stop codon followed by a restriction site for insertion into the expression vector (see Materials and methods). The various cDNA segments were linked in frame to the signal peptide sequence of human BM-40, which encompassed the signal peptidase cleavage site. This fragment was also generated by PCR and introduced an *Nhe*I site at the 3′-end for fusion.

The signal peptide sequence from the human basement membrane protein BM-40 was used because efficient expression and secretion has been observed for recombinant BM-40 using the same expression vector (Nischt *et al.*, 1991). With the largest construct (B2III3–5), we initially examined two variants which added to the N-terminal laminin sequence either a long (APQQEA) or a short (AP) sequence derived from BM-40 and a sequence (LA) due to the insertion of the *Nhe*I site. Since both variants were efficient in producing the laminin fragment all other constructs were prepared with a short additional sequence variant (Table I).

The various expression vectors were initially examined by transient transfections of human 293 embryonic kidney cells and showed expression of the expected recombinant products by Northern hybridization and/or electrophoresis of serum-free cell culture medium. Stable cell clones were then established by puromycin selection (Nischt *et al.*, 1991), which was successful for all constructs except B2III5. Northern hybridization showed the expected size of mRNA

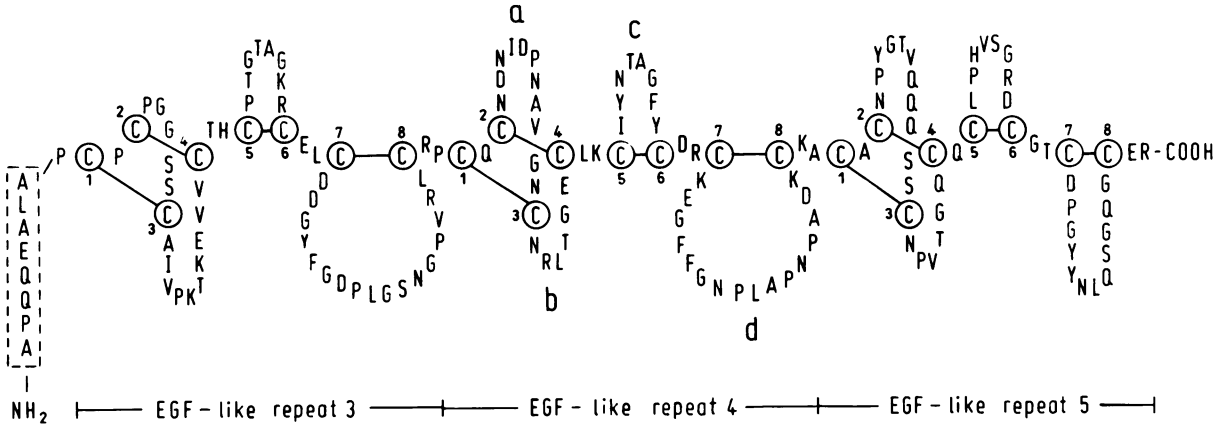

Fig. 1. Amino acid sequence of EGF-like repeats within domain III of mouse laminin B2 chain possessing the major nidogen binding site. This sequence corresponds to that of the recombinant fragment B2III3–5. The boxed N-terminal sequence orignates artificially from engineering to a signal peptide. The encircled cysteines in each repeat are numbered 1–8 and the predicted disulfide bridges (Engel, 1989) are shown by connecting lines. Individual loops generated by disulfide bonding are denoted a–d. The laminin sequence corresponds to amino acid positions 737–899 of the B2 chain (Sasaki and Yamada, 1987).

Table I. Design of recombinant fragments and their N-terminal sequence and position within laminin B2 chain domain III

Fragment[a]	Position	N-terminal sequence[b]	Primers[c]
B2III3–5	737–899	APQQEALAPCPCPGG	K23/K24
B2III3–4	737–848	APLAPCPCPGGSSXAIVPKT	K23/K27
B2III4–5	791–899	APLARPCQCNDNIDP	K31/K24
B2III3	737–792	APLAPCPCPGGSSXAI	K23/K25
B2III4	791–848	APLARPCQCNDNIDP	K31/K27
B2III5	847–899[a]		K28/K24

[a]Arabic numbers correspond to the EGF-like motifs shown in Figure 1.
[b]Determined by Edman degradation. A point identifies the beginning of the authentic laminin sequence. X, non-identified.
[c]Pairs of oligonucleotide primers (see Materials and methods) used for amplification of the corresponding laminin sequence.
[d]Only obtained after transient transfection which did not allow sequence analysis.

for B2III3−5 (~0.6 kb), B2III3−4 and B2III4−5 (~0.4 kb) and B2III3 and B2III4 (~0.2 kb) (data not shown).

Structural properties of the recombinant fragments

The five recombinant laminin fragments (B2III3−5, B2III3−4, B2III4−5, B2III3 and B2III4) could be purified from 0.5−1 l serum-free culture medium by a two step procedure. They did not bind to a DEAE-cellulose column and were then obtained from the flow through fraction in almost pure form by molecular sieve chromatography on a Superose 12 column. The final yields per 1 l culture medium were 5−8 mg for B2III3−5 and ~1−2 mg for the other fragments. Their amino acid compositions were within the limits of analytical error (± 10%) identical to that predicted from the sequence. Fragment B2III3−5 did not contain hexosamines (<0.2 residues/peptide) in agreement with the absence of carbohydrate acceptor sites. All fragments showed single N-terminal sequences by Edman degradation (Table I) which exactly matched those expected from the constructs including the residues artificially introduced at the N-terminus.

Electrophoretic analysis of the purified fragments under reducing conditions showed a single major band with only marginal contamination (Figure 2). The heterogeneity appeared somewhat higher for fragments B2III3−5 and B2III4−5 when analyzed under non-reducing conditions. These fragments contained a few more bands, representing ~10−20% of the total material, which migrated faster than the major band. We interpret these observations as an indication for the presence of variants which are incomplete in their disulfide links or have some of them incorrectly connected. This is supported by the evidence of a single N-terminal sequence for the mixture of bands and the conversion of all bands into a single one by reduction (Figure 2).

The apparent molecular mass estimated from electrophoresis was 28 kDa for non-reduced and 22 kDa for reduced B2III3−5 while the calculated mass was 18.1 kDa. A similar deviation from the theoretical mass was also observed for the smaller recombinant fragments. The anomalous migration in SDS electrophoresis agrees with previous findings for laminin P1 subfragments, which demonstrated an apparent mass contribution of 9 kDa by individual EGF-like repeats (Gerl *et al.*, 1991).

Extensive digestion of B2III3−5 with trypsin, chymotrypsin or pepsin at a 1:100 enzyme−substrate ratio revealed no substantial degradation as judged from electrophoresis under reducing and non-reducing conditions. This corresponds to previous observations of a similar resistance of the laminin P1 structure to pepsin and neutral proteases (Ott *et al.*, 1982). Digestion with proteinase K at a 1:10 ratio produced, however, a major fragment corresponding to about two EGF-like repeats.

Activity in nidogen binding assays

Previous studies by direct ligand binding and competition assays have shown that laminin and its fragment P1 bind with similar affinity to tissue-derived or recombinant nidogen (Mann *et al.*, 1988; Fox *et al.*, 1991) A 2- to 3-fold lower activity was observed for the 28 kDa B2 chain fragment B2-III-28 (Gerl *et al.*, 1991) that was obtained by pepsin digestion of partially reduced fragment P1. By using the same assays we could demonstrate a strong binding of nidogen to recombinant fragment B2III3−5 (Figure 3) with an affinity (K_d = 0.36 nM) comparable with that found for laminin fragment P1 (Fox *et al.*, 1991). ^{125}I labeling of B2III3−5 did not interfere with affinity, as shown by the high plateau level of 80% binding in the region of nidogen excess. A low plateau level of 30% binding was observed with the smaller fragment B2III4 (Figure 3). This fragment contains only two tyrosines (Figure 1) and iodine labeling may have inactivated a substantial portion of the ligand. Half maximal binding of B2III3−5 and B2III4 occurred, however, at similar nidogen concentrations, indicating that both fragments have a comparable affinity for nidogen.

All recombinant fragments were then compared with

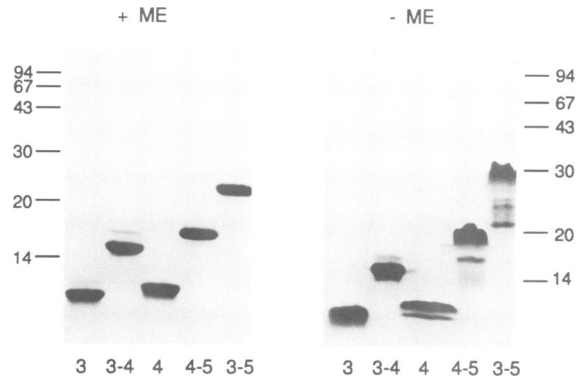

Fig. 2. Electrophoresis patterns of purified recombinant B2III fragments prior to (−ME) and after (+ME) reduction of disulfide bonds. Individual fragments are denoted by their EGF repeat numbers (Figure 1, Table I). Runs were carried out in 15% gels calibrated with reduced (left margin) or non-reduced (right margin) globular proteins. Their molecular masses are given in kDa.

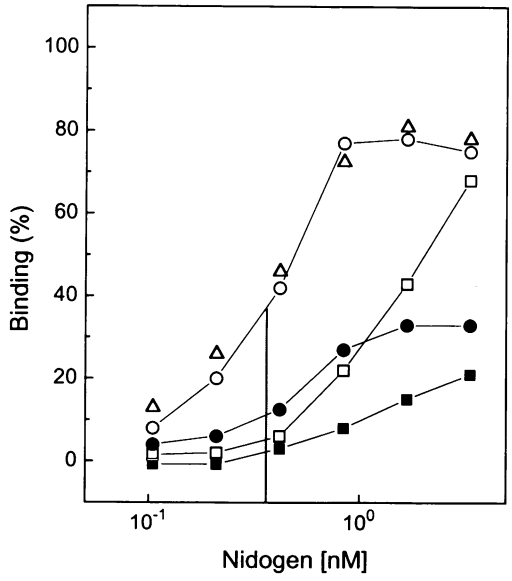

Fig. 3. Radioligand binding between recombinant nidogen and ^{125}I-labeled recombinant laminin fragments. The labeled fragments were B2III3−5 used at concentrations of 1.3 nM (□), 0.13 nM (○) and 0.013 nM (△) and B2III4 used at concentrations of 7.5 nM (■) and 0.75 nM (●). The vertical line indicates the nidogen concentration required for half maximal binding at the two lowest concentrations of B2III3−5. Since these two binding curves are superimposable, this concentration (0.36 nM) equals the apparent K_d (Engel and Schalch, 1980).

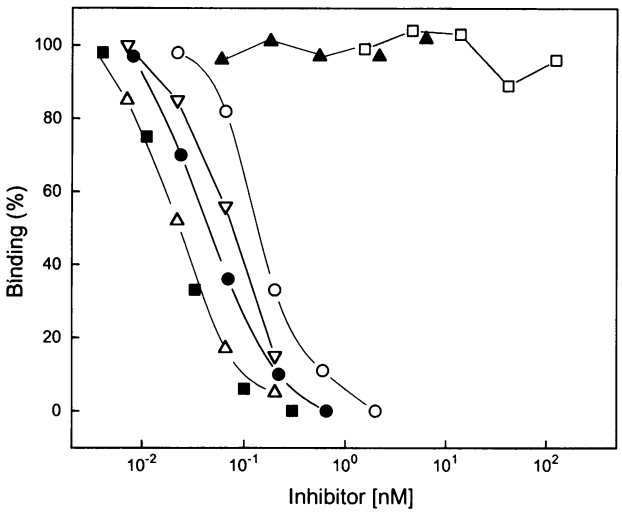

Fig. 4. Competition of nidogen binding to ^{125}I-labeled laminin fragment P1 by recombinant laminin fragments. The non-labeled inhibitors used were laminin fragment P1 (○), and recombinant fragments B2III3−5 (△), reduced and alkylated B2III3−5 (▲), B2III3−4 (■), B2III4−5 (▽), B2III3 (□) and B2III4 (●).

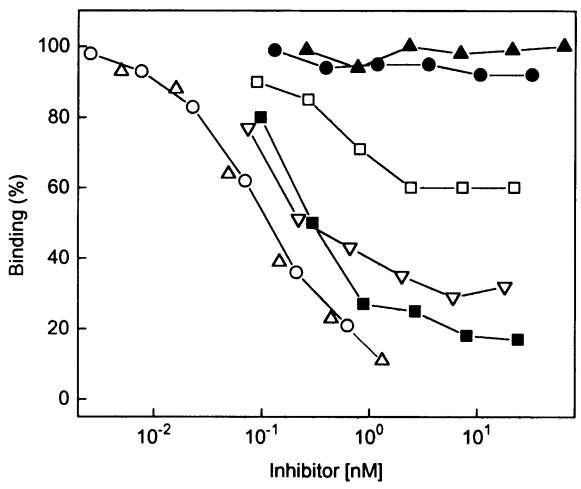

Fig. 5. Radioimmuno-inhibition assay analysis of B2III3−5 epitopes. The assay consisted of fixed concentrations of an antiserum against laminin fragment P1 and ^{125}I-labeled fragment B2III3−5. Inhibitors used were laminin fragment P1 (○) and recombinant fragments B2III3−5 (△), reduced and alkylated B2III3−5 (▲), B2III3−4 (■), B2III4−5 (▽), B2III3 (□) and B2III4 (●).

Table II. Antigen-binding capacity (ABC-33) of antisera to laminin fragment P1 and recombinant fragment B2III3−5 in radioimmunoassay

Antiserum to	ABC-33 (μg/ml) for ^{125}I-labeled fragment		
	P1	B2III3−5	B2III4
P1, 997+[a]	735	48	8
P1, 1006+[a]	630	186	<6
B2III 3−5	68	105	<2

[a]Two individual antisera identified by code Nos.

laminin fragment P1 in a competition assay (Figure 4). Fragments B2III3−5, B2III3−4, B2III4−5 and B2III4 could completely block P1-binding to nidogen and were 1.6- to 5-fold more active than fragment P1. Complete reduction and alkylation of disulfide bonds abolished the inhibitory activity of B2III3−5. Fragment B2III3 was inactive in inhibition even when used at 5000-fold higher concentrations than the active fragments. Since stable cell clones producing fragment B2III5 could not be obtained, we compared culture media obtained from transient transfections with B2III3−5 and B2III5 vectors. The media were adjusted to similar concentrations of the recombinant fragments, as estimated from electrophoresis, and demonstrated in inhibition assays that B2III5 is at least 100-fold less active than B2III3−5. Together the data show that EGF-like repeat 4 (fragment B2III4) is sufficient for nidogen binding.

Studies on the specificity of binding to nidogen were performed with immobilized fragment P1 and B2III3−5 ligands. They showed equivalent binding of soluble nidogen but no binding of the recombinant N-terminal fragment Nd-I of nidogen (data not shown). Binding of B2III3−5 to the C-terminal nidogen fragment Nd-II was shown by complex formation (see below). These observations are in complete agreement with data obtained for laminin fragment P1 (Fox *et al.*, 1991).

Immunological properties and inhibiting antibodies

Antisera against fragment P1 showed in radioimmunoassays distinct binding of fragment B2III3−5 which was 3- to 6-fold lower than P1. An antiserum raised against B2III3−5, however, bound B2III3−5 somewhat more strongly than P1. These antisera showed no or only little binding to fragment B2III4 (Table II). The data indicate that fragments P1 and B2III3−5 share major epitopes which are, however, not found on fragment B2III4.

The specificity of antibody binding was further examined by inhibition assays using an anti-P1 antiserum and ^{125}I-labeled B2III3−5 (Figure 5). Fragments P1 and B2III3−5 were of equivalent inhibitory capacity, while no activity was found for reduced and alkylated B2III3−5. Various smaller recombinant fragments except B2III4 were also active but usually showed only partial inhibitions. Studies with other antisera (not shown) revealed similar data, in particular complete inhibitions by P1 and B2III3−5 and lack of inhibition by reduced and alkylated B2III3−5. This indicates a complex repertoire of epitopes which depend on intact disulfide bonds.

Affinity-purified antibodies against B2III3−5 were also used as competitors for the binding of biotinylated nidogen to immobilized fragment P1 and B2III3−5 ligands. They showed a dose-dependent inhibition for both immobilized substrates (Figure 6) with similar IC_{50} values (5−15 μg/ml). Since binding of these antibodies to the B2III4 structure is minimal (Table II) it indicates that inhibition occurs by steric hindrance.

Complex formation and cross-linking

Since fragment B2III3−5 is of a size amenable to three-dimensional structural analyses we also examined the possibility of preparing complexes with nidogen or its laminin-binding fragments on a preparative scale. A stoichiometric mixture of B2III3−5 and nidogen could be separated from small amounts of non-bound ligands by molecular sieve chromatography and showed by electrophoresis the presence of both ligands in the same peak (Figure 7a), demonstrating the formation of a stable non-covalent complex. This complex could be readily converted into a covalent structure by the cross-linker BS^3 (see Material and methods), as shown by a shift of the

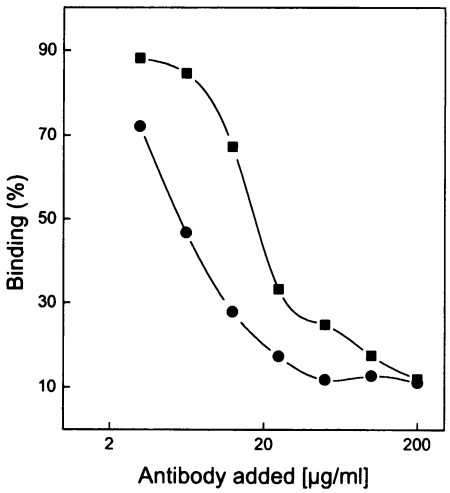

Fig. 6. Antibody inhibition of biotinylated nidogen-binding to immobilized laminin fragments P1 and B2III3−5. The immobilized coats (P1, ■; B2III3−5, ●) were pre-incubated with affinity-purified antibodies before adding nidogen (70 nM) and subsequently streptavidin−peroxidase. Control values (100% binding) were in the absence of antibodies.

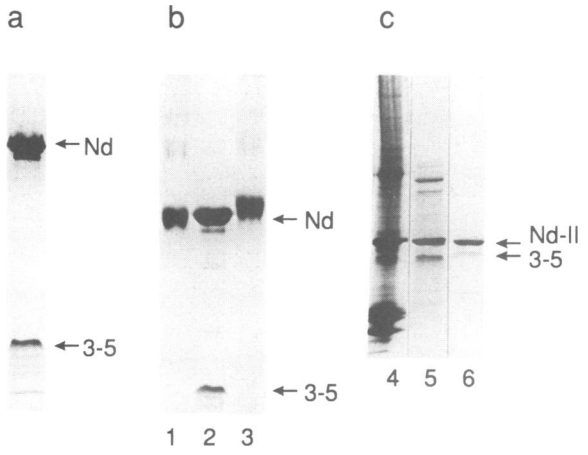

Fig. 7. Electrophoretic analysis of purified nidogen−B2III3−5 complex (**a**), of the covalent cross-linking of the complex (**b**) and of the purification of the nidogen fragment NdII complexed to BIII3−5 (**c**). Lane (a) was with a 5−15% gel showing the positions of nidogen (Nd) and the laminin fragment (3−5). Covalent cross-linking (b) is shown in a 3−12% gel. Lane 1, cross-linked nidogen; lane 2, non-cross-linked complex nidogen−B2III3−5; lane 3, cross-linked complex nidogen−B2III3−5, 8−18% gels were used for the Nd-II−B2III3−5 complex and positions for Nd-II and B2III3−5 (3−5) are indicated. Lane 4, Nd-II-containing culture medium with B2III3−5 added; lane 5, after purification of the complex on Superose 12; lane 6, after final purification on a Mono Q column.

nidogen band to a slower migrating position (Figure 7b, lanes 1−3). This shift corresponded to an increase in molecular mass of about 20 kDa. We have previously shown that adding laminin fragment P1 to culture medium containing the C-terminal recombinant nidogen fragment Nd-II led to the formation of a stable and soluble complex between both components (Fox *et al.*, 1991). This increased the poor solubility and stability of fragment Nd-II. By using the same procedure with fragment B2III3−5 we could obtain a complex of similar stability which however, required an additional Mono Q chromatography for final purification (Figure 7c, lanes 4−6).

The high protease stability of B2III3−5 also led us to investigate its cross-linked complex with nidogen by degradation with endoproteinase Glu-C. Digestion was monitored by electrophoresis and immunoblotting with antibodies against B2III3−5 and demonstrated the fast appearance of a 120 kDa followed by a broad 40 kDa band that both possessed B2III3−5 epitopes. This indicates a 10−12 kDa contribution by nidogen in the smaller component. The 40 kDa component was purified by molecular sieve and reverse phase chromatography and showed by Edman degradation a double sequence indicating the presence of two components in about equal proportions. One sequence corresponded to the N-terminus of B2III3−5 (Figure 1) and the other sequence, GIALDHLGRTIF, started at position 995 of nidogen (Mann *et al.*, 1989) within its G3 domain. The relative increase in mass of B2III3−5 and the failure to identify any other nidogen sequence indicated that the covalent complex contains a nidogen fragment corresponding to positions 995−1061 or 1087. Digestion of nidogen alone demonstrated endoproteinase Glu-C cleavage at these positions but in addition at a single peptide bond (position 1016−1017) between them. Since cleavage at this site would not fit the size estimate it indicates protection against proteolysis either by complex formation or cross-linking.

Discussion

Mouse EHS tumor laminin contains 42 EGF-like repeats which are exclusively located in its three short arms (Sasaki *et al.*, 1988; Beck *et al.*, 1990). These repeats differ from the classical EGF motifs (Apella *et al.*, 1988) by two extra cysteines (Nos 7 and 8, Figure 1) which are thought to form an additional loop d (Engel, 1989). Such laminin-type repeats are also found in the proteoglycan perlecan (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992) and the synaptic protein agrin (Rupp *et al.*, 1991; Tsim *et al.*, 1992) and therefore represent a motif frequently used in multidomain proteins. Based on previous observations that EGF-like repeats of the laminin B2 chain are required for nidogen binding (Gerl *et al.*, 1991) we have now established a versatile expression system in human cells which allows isolation of the recombinantly expressed repeats from the culture medium. The successful production of several individual as well as tandemly arranged repeats demonstrates that this structure represents an autonomous folding unit within larger proteins. The recombinant products apparently possess primarily correctly formed disulfide bridges, as shown by electrophoresis, protease resistance and the loss of binding activity for nidogen and antibodies after reduction and alkylation. Small amounts of incompletely folded material may be present in some recombinant fragments (B2III3−5, B2III4), as indicated from additional bands observed by electrophoresis. This heterogeneity had, however, no obvious effect on binding activity and did not prevent crystalization of fragment B2III3−5 (see below).

The recombinant fragment B2III3−5 was designed according to the smallest laminin fragment B2-III-28 possessing substantial nidogen binding activity (Gerl *et al.*, 1991). We included in the construct the whole EGF-like repeat 3 but not repeat 6, both of which are present in B2-III-28 as partially degraded structures. Recombinant B2III3−5 was 5- to 10-fold more active in nidogen binding

when compared with laminin fragments P1 and B2-III-28 (Figure 4, Gerl *et al.*, 1991). This excludes the participation of repeat 6 structures in the binding site and, since fragment B2-III-28 was only obtained in partially reduced form, emphasizes the crucial role of disulfide bonds for high affinity. The latter fact was underscored by showing inactivation of B2III3−5 after extensive reduction and alkylation. We could further show that repeat 4 either alone or in combination with repeats 3 or 5 possesses full binding activity. The single repeats 3 and 5 were inactive, which is explained by their low sequence identity when compared with repeat 4 (Figure 1). Furthermore, antibodies against B2III3−5 almost completely blocked nidogen binding to laminin fragment P1 which consits of 29 EGF-like repeats contributed by the A, B1 and B2 chains (Beck *et al.*, 1990; Gerl *et al.*, 1991). Together, these data leave no doubts that repeat 4 of B2 chain domain III represents the only high affinity nidogen binding site of mouse EHS tumor laminin.

Previous studies have localized the laminin binding site of nidogen to its C-terminal globular domain G3, which has an approximate diameter of 4.8 nm (Mann *et al.*, 1988; Fox *et al.*, 1991). This was confirmed here by the formation of a stable non-covalent complex between fragment B2III3−5, with an estimated length of 7.5 nm (Engel, 1989), and fragment Nd-II, which corresponds to G3. In addition, proteolytic degradation of a cross-linked nidogen−B2III2−5 complex identified a short nidogen G3 segment of either 67 or 93 residues which was covalently bound to B2III3−5. This segment is located between the first and second cysteines of nidogen domain G3 which are involved in two different disulfide bridges (Mann *et al.*, 1989; Reinhardt, 1992). It possesses two lysine residues which are apparently utilized in the covalent connection to other lysines contributed by repeats 3 and/or 4 of B2III3−5. Since the cross-linking reagent may span a distance of 1.6 nm (Geisler *et al.*, 1992) it is however, not clear whether the nidogen segment contains the binding site of G3 or is located in its close vicinity. The protection of a single protease cleavage site within the nidogen sequence favors the first possibility, but this will require further studies.

The region of the human laminin B2 chain sequence (Pikkarainen *et al.*, 1988) corresponding to the mouse B2III3−5 structure showed ~93% identity, indicating a strong conservation of the nidogen binding site. A different picture emerges from a comparison with *Drosophila* laminin B2 chain (Chi and Hui, 1989). This demonstrates a strong conservation in the EGF-like repeat 4 within loops a, b and c, but not in loop d, and also a lower level of identity for most of the repeat 3 and 5 sequences (Table III). This would predict that the *Drosophila* analog of repeat 4 may have a similar function to that in mouse laminin. Evidence for this possibility was obtained in preliminary studies, which demonstrated a strong binding of *Drosophila* laminin (Fessler *et al.*, 1987) to mouse nidogen (L.Fessler, U.Mayer and R.Timpl, unpublished). The cDNA-derived sequence of a novel human laminin B2 chain isoform (B2t) has been recently reported (Kallunki *et al.*, 1992). This variant is shorter than the B2 chain, particularly in the short arm region, and shows only a low identity (44%) in the conserved domains. Yet the B2t chain possesses an EGF-like repeat (positions 516−572) appearing as an analog of the B2 chain repeat 4 both in its position in domain III as well as in its sequence (76% identity, 10% conservative replacements). Here it will also be of interest to examine nidogen binding,

Table III. Sequence identities between mouse and *Drosophila* laminin B2 chain domain III of the EGF-like repeats 3−5

Loop	% identity in repeat		
	3	4	5
a	20	90	29
b	10	88	67
c	50	63	50
d	59	31	75

The comparison is based on individual loops (a−d, see Figure 1), not including the invariant cysteines, of cDNA sequences reported previously (Sasaki and Yamada, 1988; Chi and Hui, 1989).

which could then assist in the design of site-directed mutants of repeat 4. Circumstantial evidence from ^{125}I-labeling (Figure 3) indicates so far that one tyrosine of loop c (Figure 1) could be involved in the binding site of repeat 4.

The possibility of producing recombinant B2III3−5 in large quantities has also allowed us to examine crystals of the component, which produced distinct X-ray diffraction patterns (J.Stetefeld and R.Huber, unpublished). The elucidation of the three-dimensional structure of B2III3−5 is now in progress and may lead in the future to the elucidation of binding sites by analyzing complexes prepared from appropriate laminin and nidogen fragments. EGF-like repeats similar to those of B2III3−5 provide a cryptic, RGD-dependent cell adhesion site in the laminin A chain (Aumailley *et al.*, 1990) while others are responsible for the mitogenic activity of laminin (Panayotou *et al.*, 1989). The versatile recombinant expression system utilized here will therefore be useful to study the structural basis of other functions of laminin and related proteins.

Materials and methods

Preparation of expression vectors and transfected cells

Various fragments of the mouse laminin B2 chain cDNA (Sasaki and Yamada, 1988) were amplified by PCR (Perkin Elmer/Cetus) according to the supplier's instructions, using the full length cDNA clone pKH7 or derived subclones as templates. The oligonucleotide primers used were K23: GATCGCTAGCGCCTTGTCCCTGCCCC; K24: GATCTCTAGACTCGAGCTACCTCTCGCAGCCTTGC; K25: GATCCTCGAGCTACGGGCGGCACAGTCTC: 31: GATCGCTAGCACGCCCGTGCCAGTGTAAC; K27: GATCCTCGAGCTAGGCTTTGCATTTGTCG; and K28: GATCGCTAGCCAAAGCCTGCGCCTGCA. Oligonucleotides K23, K28 and K31 contain an additional *Nhe*I site at the 5′-end and oligonucleotides K24, K25 and K27 a stop codon followed by a *Xho*I site at the 3′-end. They were used in different combinations to encompass different EGF-like repeats (Table I). The largest 519 bp fragment, B2III3−5, was produced in a reaction mixture (100 μl) containing 10 ng pkH7, 1 μg K23, 1.5 μg K24, 200 nM each of dATP, dCTP, dGTP and TTP, 2.5 units Taq polymerase (Stratagene), 10 mM Tris−HCl pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$, which was cycled 25 times at 94°C (15 s), 53°C (20 s) and 72°C (120 s). The product was purified by gel electrophoresis, phosphorylated with polynucleotide kinase and, after addition of a *Cla*I linker, cloned into *Cla*I restricted pBluescript vector to yield vector pB1B2. The other PCR products were prepared with pB1B2 as template, digested with *Nba*I/*Nhe*I and cloned into pB1B2 restricted with the same enzymes. All PCR products were verified by DNA sequencing.

Fragments corresponding to the 5′-end of human BM-40 cDNA (Lankat-Buttgereit *et al.*, 1988) were generated by PCR amplification with clone pBM-40 (Nischt *et al.*, 1991) as template and the oligonucleotides Ti1: GATATCTAGAAGCTTCTGCCTGCCGCCTG; Ti2: GATCGCTAGCGCTTCTTGCTGAGG; and Ti3: GATCGCTAGCTCTCACTGAAACA as primers. The oligonucleotides introduce additional *Nhe*I sites at the 3′-end (Ti2, Ti3) or *Xba*I and *Hin*dIII sites at the 5′-end (Ti1) and were used in two combinations to yield a 126 bp fragment Ti1−Ti3 and a 138 bp fragment Ti1−Ti2. The fragments encode portions of the 5′-non-coding region (44 bp), the whole signal peptide and four (Ti1−Ti3) or eight (Ti1−Ti2) more amino acids beyond the cleavage site, including two foreign

amino acids at the 3'-end introduced by generating the *NheI* restriction site. The PCR products were cut with *XbaI* and *NheI*, purified by gel electrophoresis and combined with *NheI*/*XhoI* restricted laminin-encoding fragments (see above) within the *XbaI*/*XhoI* restricted eukaryotic expression vector pCis (Gorman *et al.*, 1990). The correct ligation and in frame insertion of both fragments was verified by DNA sequencing.

The various new expression vectors were then used for transient transfections of human embryonic kidney cells 293 and for stable transfections by puromycin selection following previously described procedures (Nischt *et al.*, 1991). Transfected cells were initially analyzed by Northern hybridization and by SDS electrophoresis of serum-free culture medium. Larger volumes of serum-free cell culture medium were then prepared for protein purification (Nischt *et al.*, 1991; Fox *et al.*, 1991).

Protein purification and analysis

Culture medium (0.5–1 l) from cell clones was dialyzed against 0.05 M Tris–HCl, pH 8.6 and passed over a DEAE–cellulose column (2.5 × 20 cm) equilibrated in the same buffer. Proteins which did not bind to the column were dialyzed against 0.2 M NH_4HCO_3 and lyophilized. They were then dissolved in 0.2 M ammonium acetate pH 6.9 and passed over a Superose 12 column (HR 16/50, Pharmacia). Laminin fragment P1 was prepared from EHS mouse tumor material by pepsin digestion (Ott *et al.*, 1982; Aumailley *et al.*, 1990). Recombinant mouse nidogen and its fragments Nd-I and Nd-II were obtained following previously described procedures (Fox *et al.*, 1991).

Amino acid and hexosamine compositions were determined on an LC 5001 analyzer (Biotronik) after hydrolysis with 6 M or 3 M HCl (16 h, 110°C) respectively. Cysteines were determined after reduction and *S*-carboxymethylation. Complete reduction and alkylation of disulfide bonds was achieved in 6 M guanidine–HCl (Gerl *et al.*, 1991). SDS electrophoresis was carried out in polyacrylamide gradient gels by established protocols. Edman degradation was performed with gas phase sequencer models 470A and 473A (Applied Biosystems) following the manufacturer's instructions. Proteolytic digestions (enzyme–substrate ratios 1:10 or 1:100) were carried out for 24 h at 37°C using trypsin, chymotrypsin or proteinase K in 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 2 mM *N*-ethylmaleimide, 2 mM $CaCl_2$ or pepsin in 100 mM glycine–HCl, pH 1.9.

Preparation of protein complexes

Recombinant nidogen and fragment B2III3–5 dissolved in 0.2 M NH_4HCO_3 were mixed for 24 h, 4°C at a final concentration of 9–10 μM and then passed over a Superose 12 column in 0.2 M ammonium acetate, pH 6.9. The complex was lyophilized and dissolved in 20 mM triethanolamine pH 8.0, 150 mM NaCl (~1 mg/ml), then cross-linked with bis(sulfosuccinimidyl) suberate (BS^3, Pierce) and the reaction was stopped by adding an excess of ethanolamine (Geisler *et al.*, 1992). The preparation of a complex between nidogen fragment Nd-II present in culture medium and B2III3–5 basically followed a previous protocol (Fox *et al.*, 1991). After concentration to ~0.6 ml and Superose 12 (HR 10/30, Pharmacia) chromatography the peak containing the complex was chromatographed on a Mono Q column (Mayer *et al.*, 1991). Non-complexed B2III3–5 did not bind to the column while the complex was eluted with 0.08 M NaCl.

Ligand binding and immunological assays

Protein interactions were analyzed by a radioligand assay with both reactants in the fluid phase. Bound radiolabeled ligands were then coprecipitated with an excess of antibodies against the non-labeled ligand (Mann *et al.*, 1988; Gerl *et al.*, 1991). In a few cases assays with one ligand in immobilized form were also applied (Aumailley *et al.*, 1989). In other assays independent of an antibody detection system, nidogen was used after biotinylation with NHS-biotin (Pierce) following the manufacturer's instructions. Binding of the product to immobilized ligands was then detected by incubation with streptavidin–peroxidase (Sigma) used at 1 μg/ml for 90 min at room temperature.

Preparation of antisera, radioimmunobinding and inhibition assays and affinity purification of antibodies followed established protocols (Timpl, 1982). An affinity column with laminin fragment P1 was used to purify antibodies from an antiserum against B2III3–5. Antigen-binding capacity (ABC-33) was calculated from radioimmunoassays (Timpl, 1982). Immunoblotting was performed after electrophoretic separation and transfer to Immobilon membranes following the manufacturer's instructions (Biometra).

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