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Protein binding and cell adhesion properties of two laminin isoforms (AmB1eB2e, AmB1sB2e) from human placenta

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SUMMARY

Two isoforms of laminin were extracted from human placenta by neutral buffer containing EDTA, copurified through several steps and finally separated by Mono Q anion exchange chromatography. One variant consisted of disulphide-linked 340, 230 and 190 kDa subunits, which were identified by immunoblotting as Am, B1e and B2e chains. In the other variant, the B1e chain was replaced by B1s of 180 kDa. After rotary shadowing, both variants showed a similar cross-shaped structure. The nidogen content of these laminins was substoichiometric and variable (3-70%), indicating loss by endogenous proteolysis. Yet both human isoforms were able to bind mouse nidogen with an affinity (K_d~0.5 nM) comparable to that of AeB1eB2e laminin from a mouse tumour. Since the binding site is known to be contributed by a single EGF-like motif of the B2e chain, this demonstrates that activity of this site is independent of chain assembly. Binding activity of both isoforms to collagen IV and the heparan sulphate proteoglycan perlecan was correlated to the nidogen content and could be enhanced by adding nidogen. Binding to heparin was only partial and heparin did not inhibit perlecan binding. This indicated a crucial role for nidogen in mediating the integration of these laminin isoforms into basement membranes. Variant AmB1sB2e showed calcium-dependent binding to fibulin-1, while only a little activity was found for AmB1eB2e. Both isoforms promoted adhesion and spreading of several cell lines. Adhesion could be completely inhibited by antibodies to the integrin β_1 subunit but not, or only weakly, by antibodies against β_3, α_2, α_3, α_5 and α_6 subunits. No inhibition was observed with an Arg-Gly-Asp-containing peptide.

Key words: basement membrane, protein interaction, integrin, nidogen, fibulin-1

INTRODUCTION

Laminin and collagen IV are the major constituents of basement membranes, where they exist as multiple isoforms with different distributions (Paulsson, 1993). A prototypic laminin variant was originally obtained from the mouse Engelbreth-Holm-Swarm (EHS) tumour (Timpl et al., 1979) and shown to consist of three disulphide-linked chains (B1e, ~200 kDa; B2e, ~200 kDa; Ae, ~400 kDa) that assemble into a cross-shaped molecule (Beck et al., 1990; Engel, 1993). The N-terminal regions of the three chains form the three short arms of laminin and the C-terminal regions join to form a triple-coiled α helix, which directs chain assembly (Hunter et al., 1990, 1992). Many diverse functions have been attributed to this multidomain protein, including promotion of cell adhesion and migration, maintenance of cellular phenotype, control of development, cellular proliferation and gene expression. These functions are in part mediated by binding to integrin receptors (Mecham, 1991; Engel, 1993; Kleinman et al., 1993; Ekblom, 1993). Laminin also participates in the supramolecular organization of basement membranes as shown by its self-assembly into large networks (Yurchenco and Schittny, 1990; Yurchenco et al., 1992) and heterotypic binding to other ligands. High-affinity binding to the 150 kDa protein nidogen seems to be of particular importance (Paulsson et al., 1987; Fox et al., 1991) and has been localized to a single EGF-like motif in the B2e chain (Gerl et al., 1991; Mayer et al., 1993). This reaction facilitates the formation of ternary complexes with collagen IV and the heparan sulphate proteoglycan perlecan (Aumailley et al., 1989a; Fox et al., 1991; Battaglia et al., 1992). Further binding reactions with BM-90/fibulin-1 (Pan et al., 1993) and the heparan sulphate side-chains of perlecan (Battaglia et al., 1992) are mediated by globular domains at the C-terminal end of the Ae chain.

Several observations during the last 10 years have indicated that the mouse tumour-derived laminin represents only one member of a large family of similar proteins. The most compelling evidence came from cDNA sequence analyses that identified an Am chain (merosin) homologous to the Ae chain (Ehrig et al., 1990) and a B1s chain (referred to previously as s-laminin; Hunter et al., 1989) homologous to the B1e chain (for nomenclature see Engel et al., 1991; Paulsson, 1993). Tissue localization and affinity chromatography studies with antibodies specific for the five known laminin chains showed

that at least four isoforms should exist, with the chain compositions AeB1eB2e, AeB1sB2e, AmB1eB2e and AmB1sB2e (Engvall et al., 1990; Sanes et al., 1990). The data also indicated that the presence of Ae and Am within individual laminin molecules is mutually exclusive, as is the case for B1e and B1s chains, which is in agreement with predictions from the calculation of ionic interaction potentials within the triple-coiled α-helical assembly domain (Engel et al., 1991; Beck et al., 1993). Am-containing isoforms have been isolated and partially characterized from heart and placenta (Paulsson and Saladin, 1989; Paulsson et al., 1991; Engvall et al., 1992) and a complex mixture of laminin chains in placenta has been indicated in other studies (Ohno et al., 1983; Brown et al., 1990). Further laminin isoforms that have recently been identified include kalinin and K-laminin, which form a covalent complex in the anchoring filaments of some basement membranes (Rousselle et al., 1991; Marinkovich et al., 1992; Burgeson, 1993). A novel B2t chain, which is a truncated isoform of B2e (Kallunki et al., 1992), could be one of the constituents involved in this complex. Further preliminary evidence exists for additional A and B1 chain isoforms (Tokida et al., 1990; Seebacher et al., 1991; O'Rear, 1992). This has raised the intriguing question as to whether all laminin variants have equivalent functions. The available data are so far scarce and have been mainly limited to cellular studies (Rouselle et al., 1991; Brown and Goodman, 1991; Engvall et al., 1992).

Our present study is based on previous observations that a laminin preparation obtained from neutral salt extracts of human placentae consisted of at least five different chains (Brown et al., 1990). We have now purified from this preparation two different isoforms that share the Am and B2e chains but differ in the presence of either the B1e or B1s chain. While they were remarkably similar in their binding to most proteins and in their cell adhesion activity, they differed in some aspects from the AeB1eB2e isoform and only the B1s-containing isoform was able to bind fibulin-1(BM-90).

MATERIALS AND METHODS

Purification and separation of laminin isoforms

Laminin was purified from human placentae according to a previous procedure (Brown et al., 1990) with some modifications. Placental proteins were extracted with a neutral buffer, precipitated twice with 5 M NaCl, then subjected to heparin-Sepharose chromatography as described for the purification of collagen XIV (Brown et al., 1993). Approximately 57% of the laminin was found in the unbound fraction (pool H1) and approximately 31% in the fractions eluting between 0.2 M and 0.3 M NaCl in pools H2 and H3 (Brown et al., 1993). These unbound and eluted laminin fractions were purified separately. The pools were dialysed against 2 M urea, 20 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.3 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM sodium azide, then passed over a Whatman DE-52 column equilibrated in the same buffer. The column was eluted with a linear NaCl gradient (0.02 to 0.5 M NaCl) and eluted fractions were analysed by electrophoresis. Those containing laminin isoforms were pooled and dialysed against 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl, 3 mM PMSF, 10 mM sodium azide.

At this stage different methods were used to remove residual fibronectin contamination from the heparin binding and non-heparin binding material. The latter was passed over a 1 ml HiTrap heparin column (Pharmacia Biosystems GmbH, Freiburg, FRG) and the majority of the laminin remained in the unbound fraction. The heparin binding sample was passed over a 1 ml gelatin HiTrap column (prepared by conjugating 4.1 mg denatured bovine type I collagen to a 1 ml NHS-activated HiTrap column from Pharmacia according to the manufacturer's instructions) and again the laminin isoforms remained in the unbound fraction. The isoforms were then separated from one another by passing each of the samples over a Mono Q column (HR5/5; Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 M NaCl. The column was eluted with a linear NaCl gradient (0.1 to 0.6 M NaCl, 30 ml). In some preparations the gradient was held constant at NaCl concentrations of 0.19 M, 0.23 M, 0.26 M and 0.29 M until the absorbance returned to baseline, in order to achieve a better separation of the isoforms. Eluted isoforms were pooled separately, concentrated in Centricon-30 microconcentrators (Amicon GmbH, Witten, FRG), then size fractionated on a Superose 6 column (HR10/30 or HR16/50; Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl.

Preparation of other proteins

The laminin-nidogen complex, the laminin fragments P1 and E8, collagen IV and a heparan sulfate proteoglycan (perlecan) were prepared from the mouse EHS tumour following established protocols (Timpl et al., 1987). The same tumour was used to purify BM-90/fibulin-1 (Kluge et al., 1990). Recombinant mouse nidogen was prepared from the culture medium of stably transfected human cell clones (Fox et al., 1991). A similar procedure was used to obtain recombinant human nidogen (R. Nischt, U. Mayer and R. Timpl, unpublished data). The recombinant fragment B2III3-5 from the laminin B2e chain was obtained as previously described (Mayer et al., 1993).

Sources of antibodies and immunological assays

Rabbit antisera specific for the laminin B1e and B2e chains were raised against β-galactosidase-laminin fusion proteins XLB1.2 and XLB2.1, respectively (Brown et al., 1990). Monoclonal antibody C4 against the B1s chain (Hunter et al., 1989) was kindly provided by J. Sanes. A rabbit antiserum specific for the Am chain was raised against the 300 kDa band of mouse heart laminin (Paulsson and Saladin, 1989) and kindly provided by M. Paulsson. An antiserum specific for the Ae chain was raised against fragment E3 of murine tumour laminin and used in affinity-purified form (Klein et al., 1988). Rabbit antisera were also raised against a mixture of AmB1eB2e and AmB1sB2e laminins obtained from human placenta (Brown et al., 1990), and against recombinant murine (Fox et al., 1991) and human nidogen (R. Nischt, U. Mayer and R. Timpl, unpublished). Other antisera used in protein ligand assays were those described in previous studies (Kluge et al., 1990; Fox et al., 1991; Battaglia et al., 1992). Monoclonal antibody 3E3 against human fibronectin was purchased from Biomol Feinchemikalien GmbH, Hamburg, FRG. Monoclonal antibodies against integrin subunits including AIIB2 (β_1), C17 (β_3), Gi9 (α_2), P1B5(α_3), BIIG2 (α_5) and GoH3 (α_6) were those used in a previous study (Pfaff et al., 1993) and either obtained from commercial sources (Biomol or Dianova, Hamburg, FRG) or kindly supplied by C. H. Damsky and A. Sonnenberg.

Radioimmuno-inhibition assays for the quantitative determination of protein concentrations were carried out according to standard protocols (Timpl, 1982). Electroblotting and immunodetection of transferred proteins was carried out as previously described (Brown et al., 1990), except that the transfer buffer used was 10 mM disodium tetraborate and the peroxidase substrate consisted of 3 mg/ml 4-chloro-1-naphthol in cold methanol, which was added to 0.018% H_2O_2 in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, shortly before the detection reaction.

Analytical methods and rotary shadowing

Amino acid compositions and protein concentrations were determined after hydrolysis with 6 M HCl (110°C, 16 hours) on a LC 5001 analyser (Biotronik, Maintal, FRG). SDS-polyacrylamide gel electrophoresis was in 3% to 10% gels and was followed by electroblot-

ting or by staining with Coomassie Brilliant Blue R250. Samples were analysed nonreduced or after reduction with 2-mercaptoethanol and compared with a set of globular standard proteins including reduced laminin-nidogen complex. Samples dissolved in 0.2 M NH_4HCO_3 were used to visualize protein shapes by rotary shadowing (Paulsson et al., 1987).

Protein binding and cell adhesion assays

A radioligand assay with both reactants in soluble form has been previously described (Mann et al., 1988; Fox et al., 1991). Binding profiles were determined with ^{125}I-labelled ligands at different concentrations in order to estimate apparent K_d values (Engel and Schalch, 1980). In competition assays, a fixed amount of non-labelled ligand was preincubated with inhibitors (24 hours, 4°C) prior to the addition of the labelled ligand. Protein-protein interactions were also determined with one ligand immobilized to a plastic surface, following a previously described procedure (Aumailley et al., 1989a) with some modifications (Brown et al., 1993). In the analyses with fibulin-1, 2 mM $CaCl_2$ or 10 mM EDTA was included in the assay buffer and wash buffer (Pan et al., 1993). All binding reactions were set up in duplicate or triplicate and showed less than 20% variation.

Cell adhesion assays were as described previously (Aumailley et al., 1989b). In inhibition assays cells were preincubated with monoclonal antibodies or synthetic peptides before being added to the wells (Pfaff et al., 1993). Synthetic GRGDS and REGS were obtained from commercial sources.

RESULTS

Purification and characterization of two laminin isoforms

Human placental laminin as purified previously (Brown et al., 1990) gave rise to five bands on reducing SDS-polyacrylamide gel electrophoresis, with molecular masses of 400, 340, 230, 190 and 180 kDa. The 230 kDa and 190 kDa bands were identified as the B1e and B2e subunits, respectively. In the work described here, the purification procedure was amended. The first chromatography step involved passage over a heparin-Sepharose column, which separated collagen XIV from the human laminin (Brown et al., 1993) and in addition divided the laminin into heparin-binding and non-heparin binding fractions. In general, between one third and one half of the laminin bound to the heparin column, as measured by radioimmunoassay (see Brown et al., 1993). Both laminin fractions were purified further, but in subsequent experiments no significant structural or functional differences were found between the heparin-binding and non-heparin binding laminins (see below). After passage over DEAE-cellulose and removal of fibronectin by heparin or gelatin affinity chromatography (see Materials and Methods), the laminin-containing samples were further fractionated on a Mono Q anion exchanger. Elution with a NaCl gradient partially separated the laminin into different populations and, by holding the NaCl gradient as each peak eluted, a better separation could be achieved. A typical Mono Q profile is shown in Fig. 1, although the relative sizes of each peak varied between runs. Electrophoresis of the pooled fractions indicated that two different laminin isoforms had been separated and were present in pure form in pools B and D (Fig. 2). Pool C contained a mixture of the two isoforms, pool A contained the same isoform as pool B as well as other non-laminin material, and pool E contained a mixture of the pool D isoform and collagen XIV.

After a final purification step over Superose 6, the laminin isoform in pool B gave rise to major electrophoretic bands of 340 kDa, 190 kDa and 180 kDa and that in pool D to bands of 340 kDa, 230 kDa and 190 kDa under reducing conditions (Fig. 2A). Both isoforms also contained a band of 80 kDa and a minor band of about 600 kDa. Under non-reducing conditions, each sample gave rise to a major high molecular mass band that migrated slightly faster in pool B than in pool D, as well as a fainter band of about 67 kDa (Fig. 2B). Occasionally a band comigrating with EHS tumour nidogen (150 kDa) was observed on Coomassie Blue-stained gels, but it never reached stoichiometric proportions. Subsequent measurement of nidogen content by radioimmuno-inhibition assay showed that the molar amount of nidogen varied considerably between preparations (3-70%), indicating that it may be susceptible to proteolysis.

In order to identify the laminin isoforms present in each of the purified samples, immunoblots were carried out on pools B, C and D, using antisera or monoclonal antibodies specific for individual chains (Fig. 3). The laminin in pool B contained Am (340 kDa), B1s (180 kDa) and B2e (190 kDa) chains and that in pool D Am, B1e (230 kDa) and B2e chains. Pool C contained all four of these chains. A very low level of cross-reactivity of the anti-B1s antibody with pool D was also seen (Fig. 3D, lane 3). The 80 kDa band observed in pools B and D (Fig. 2A) probably corresponds to the non-covalently associated 80 kDa component of the Am chain (Ehrig et al., 1990) and appears to migrate slightly faster under non-reducing conditions. None of the pools cross-reacted with an antiserum specific for the laminin Ae chain (Fig. 3A) and none contained fibronectin (not shown). Thus, the laminin isoforms each

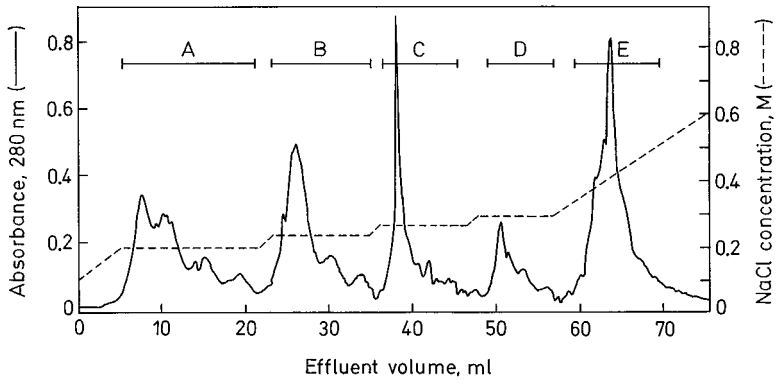

Fig. 1. Chromatographic separation of human laminin isoforms. Partially purified laminin from human placentae was passed over a Mono Q column, then eluted with a 0.1 to 0.6 M NaCl gradient (broken line), which was held constant as each peak eluted. Pools A-E (horizontal bars) were examined by electrophoresis and immunoblotting (Figs 2, 3).

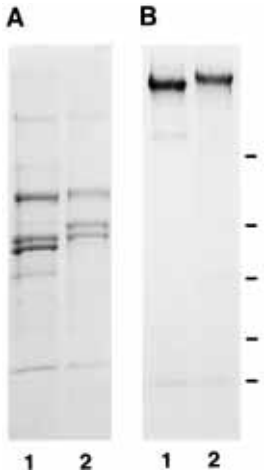

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified human laminin isoforms under reducing (A) and non-reducing (B) conditions. The samples were isoforms AmB1sB2e (pool B in Fig. 1, lanes 1) and AmB1eB2e (pool D in Fig. 1, lanes 2) after purification on Superose 6. The positions of molecular mass standards are indicated in the right margin. They were, from top to bottom; reduced Ae (440 kDa) and B1e/B2e chains (~220 kDa) of EHS tumour laminin; EHS nidogen (150 kDa); phosphorylase *b* (94 kDa); and bovine serum albumin (67 kDa). Staining was with Coomassie Blue.

consist of three different disulphide-linked chains, and will be referred to from here on as AmB1eB2e and AmB1sB2e (Paulsson, 1993). Typical yields were 200-400 μg of each purified isoform from one placenta (about 500 g). In the case of the non-heparin binding laminin, individual B chains could not be clearly distinguished by protein staining, but immunoblots indicated that the AmB1eB2e and AmB1sB2e isoforms had again been separated (not shown).

The amino acid compositions of the two human laminins were identical within the range of analytical error and were similar to that reported for mouse EHS tumour laminin (Timpl et al., 1979). Rotary shadowing of the human laminin preparations showed no detectable differences between the two isoforms. In both cases, mainly cross-shaped but also a few Y-shaped molecules were observed (Fig. 4). In all cases the globule at the end of the long arm was present, indicating that the 80 kDa component of the Am chain remained associated. Associates between nidogen and human laminin were rarely observed, while such structures are abundant in the laminin-nidogen complex isolated from the EHS tumour (Paulsson et al., 1987).

Nidogen binding

The nidogen-binding site on EHS tumour laminin has been localized to a single EGF-like motif in the B2e chain (Gerl et al., 1991; Mayer et al., 1993). Since both of the human laminin isoforms contained a B2e chain but were purified with substoichiometric amounts of nidogen, it was of interest to see whether they retained the capacity to bind to nidogen. Both of the human isoforms were able to fully inhibit the binding of nidogen to radiolabelled EHS laminin P1 fragment (Fig. 5). The molar amount required for equivalent levels of inhibition was the same for both human isoforms and approximately twofold higher than that for the P1 fragment. Direct binding was also demonstrated in ligand assays with ^{125}I-labelled human laminin isoforms and non-labelled mouse nidogen (not shown), with an estimated K_d of 0.5 nM for both interactions. A similar dissociation constant has been previously found for nidogen binding to fragment P1 (Fox et al., 1991). The plateau levels of binding at nidogen excess were, however, lower with the two human laminin isoforms (25-35%) when compared to fragment P1 (60-80%). This is obviously due to residual endogenous nidogen, which blocks some of the binding sites on the human laminin molecules.

Soluble nidogen was also shown to bind strongly to both human laminin isoforms when they had been immobilized on plastic surfaces. This interaction could be completely blocked with the recombinant fragment B2III3-5 (18 kDa), which contains the nidogen-binding EGF-like motif of the mouse laminin B2e chain (Mayer et al., 1993). The inhibitory capacity of B2III3-5 (IC_{50}=1-2 nM) was similar for both the interactions with human laminins and fragment P1. This strongly indicates that the two laminin isoforms possess only a single high-affinity nidogen binding site.

Nidogen-mediated binding to perlecan and collagen IV

Other well-characterised ligands of EHS tumour laminin (AeB1eB2e) are the heparan sulphate proteoglycan perlecan and collagen IV (Aumailley et al., 1989a; Battaglia et al.,

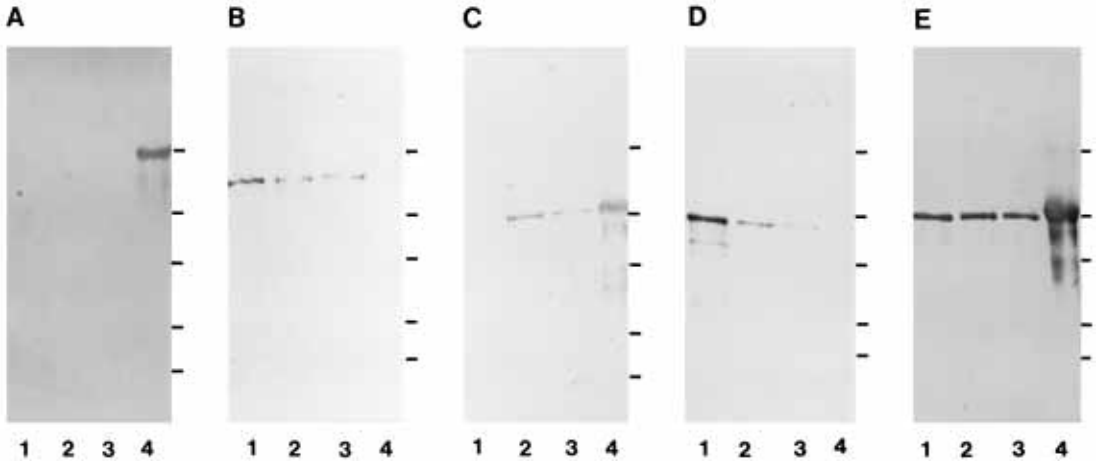

Fig. 3. Characterization of laminin isoforms by immunoblotting with chain-specific antibodies. The samples were human isoforms AmB1sB2e (lanes 1), AmB1eB2e (lanes 3), a mixture of both isoforms (pool C in Fig. 1, lanes 2) and the EHS tumour laminin AeB1eB2e (lanes 4). They were probed with antibodies against the Ae chain (A), Am chain (B), B1e chain (C), B1s chain (D) and B2e chain (E). The molecular mass standards (right margins) were as in Fig. 2. No reaction was observed with antibodies to fibronectin (data not shown).

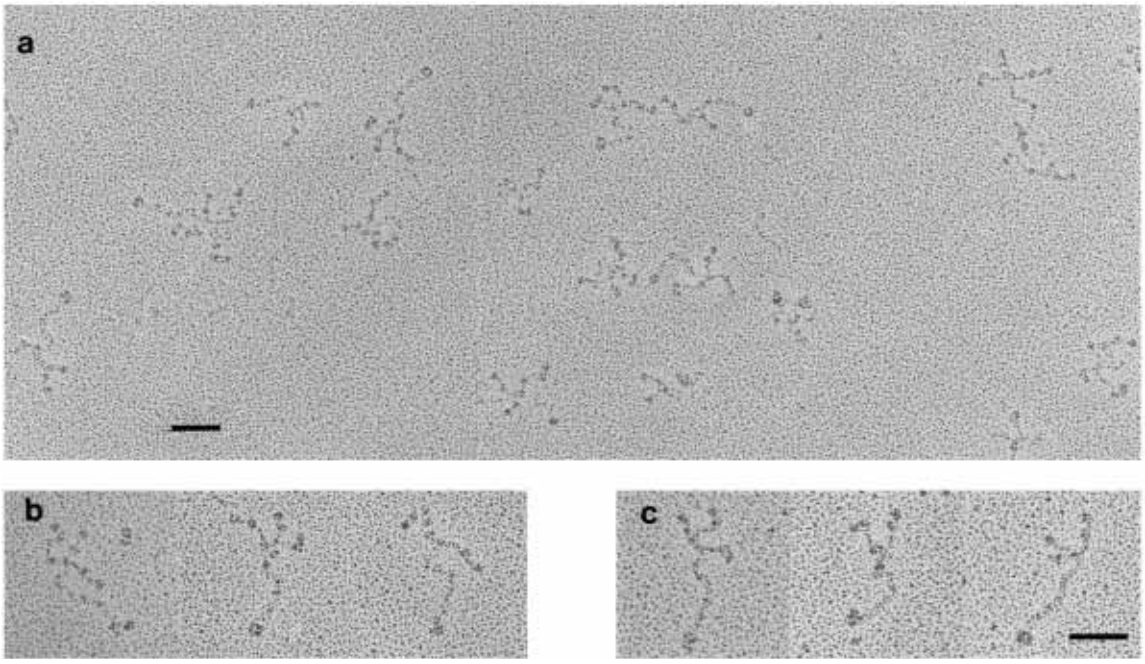

Fig. 4. Electron microscopic visualization of human laminin isoforms after rotary shadowing. (a) Representative field of isoform AmB1eB2e; (b) individual molecules of isoform AmB1eB2e; (c) individual molecules of isoform AmB1sB2e. Bars, 50 nm.

1992). In the case of perlecan, binding takes place both directly, via a site in the Ae chain, and indirectly, using nidogen as a bridging molecule. Mediation by nidogen may be the only mechanism in the binding to collagen IV (Aumailley et al., 1993). The ability of the two human laminin isoforms to bind to perlecan and collagen IV, and the role of nidogen in this process, were therefore examined.

The level of binding of perlecan to human laminin varied considerably between batches (Fig. 6) and was independent of which isoform (AmB1sB2e or AmB1eB2e) was used or whether the isoforms had bound to heparin-Sepharose or not. Further evidence that the level of binding to perlecan was independent of the capacity to bind heparin was provided by the failure of heparin (up to 500 μg/ml) to inhibit binding of those isoforms that bound well to perlecan (not shown). When the nidogen content of the different batches of human laminin isoforms was measured, however, it was found to correlate well with the perlecan-binding ability (Fig. 6). In addition, incubation of the laminin substrate with exogenous nidogen before addition of perlecan resulted in uniformly higher levels of binding (Fig. 7). In the case of collagen IV, binding of the isoforms was similarly variable and also not dependent on which isoform was being used or its heparin-binding capacity. Again, the level of binding correlated well with nidogen content and could be enhanced for the less well binding batches by addition of exogenous nidogen (Fig. 8). Thus, binding of the human laminin isoforms to perlecan and collagen IV appears not to take place directly, but in both cases is mediated via nidogen as a bridging molecule.

Differences in fibulin-1 binding of the laminin isoforms

Laminin from the EHS tumour has also recently been shown to bind to fibulin-1 (BM-90) in a calcium-dependent manner, an interaction that is thought to be mediated via a site in the laminin Ae chain (Pan et al., 1993). Since neither of the human laminin isoforms contains an Ae chain, it was of interest to see if they could bind to fibulin-1. The isoform AmB1sB2e bound to fibulin-1 in the presence of 2 mM $CaCl_2$ and the binding was greatly reduced in the presence of 10 mM EDTA (Fig. 9). AmB1eB2e showed a 50- to 60-fold lower binding to fibulin-1, which was also sensitive to EDTA. This could indicate a lower affinity but could also be accounted for by a minor contamination (~2%) of the preparation with AmB1sB2e isoform, as seen by immunoblotting (see Fig. 3D, lane 3).

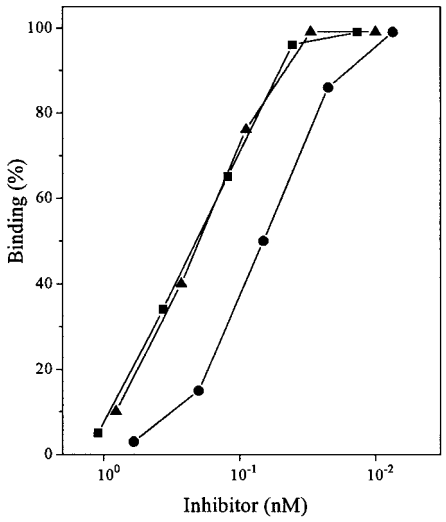

Fig. 5. Inhibition of nidogen-binding to ^{125}I-labelled laminin fragment P1 by human placental laminins. Serial dilutions of inhibitors were incubated overnight with recombinant mouse nidogen prior to addition of ^{125}I-labelled fragment P1. Samples were immunoprecipitated with an antiserum to mouse nidogen. Results are expressed as % of precipitated label in the absence of inhibitors. Inhibitors used were the laminin isoforms: AmB1eB2e (■) and AmB1sB2e (▲), and laminin fragment P1 (●) from the EHS tumour laminin AeB1eB2e.

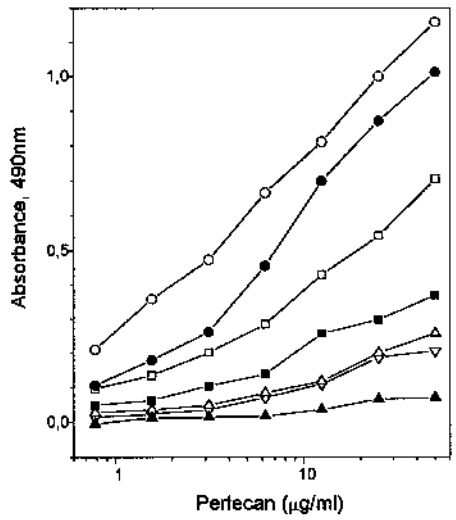

Fig. 6. Binding of perlecan to human laminin isoforms. Serial dilutions of perlecan were added to immobilized laminin and binding was demonstrated with an antiserum specific for perlecan followed by a peroxidase-labelled second antibody. After addition of peroxidase substrate, absorbance at 490 nm was measured. Different batches of laminin were compared including the purified isoforms: AmB1sB2e (●,▲), AmB1eB2e (■), and mixtures of the two isoforms (○,□,▽,△). These batches differed in the molar amounts of endogenous nidogen as measured by radioimmunoassay: ○, 69%; ●, 53%; □, 43%; ■, 35%; ▲, 26%▽, 18%; △, 15%. Note the correlation between binding activity and nidogen content.

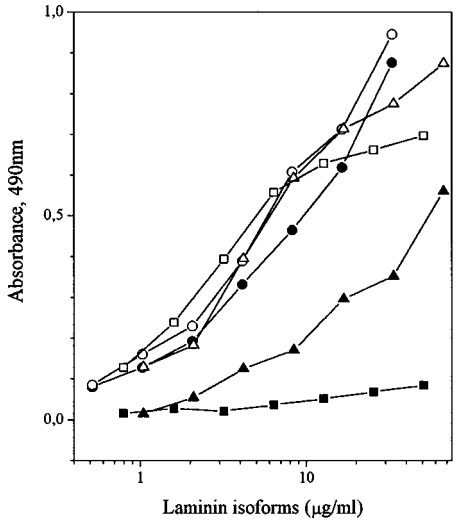

Fig. 8. Enhancement of human laminin binding to immobilized collagen IV by exogenous nidogen. Wells coated with collagen IV were incubated in the presence (open symbols) or absence (filled symbols) of mouse nidogen (10 μg/ml) before addition of AmB1sB2e (53% endogenous nidogen; ○,●), AmB1eB2e (35% nidogen; △,▲) or a mixture of isoforms (18% nidogen; □,■). Binding was detected with an antiserum against fragment P1 of EHS laminin, which reacted equally well with each human laminin isoform, followed by a peroxidase-labelled second antibody and colorimetric reaction as in Fig. 6.

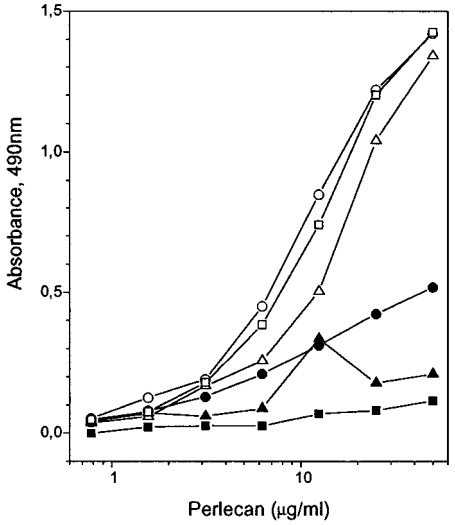

Fig. 7. Enhancement of perlecan binding to human laminin isoforms by exogenous nidogen. Plates were coated with AmB1sB2e containing 53% endogenous nidogen (○,●), AmB1eB2e (35% nidogen; △, ▲) or a mixture of both isoforms (18% nidogen; □,■) and preincubated in the presence (open symbols) or absence (filled symbols) of an excess (10 μg/ml) of mouse nidogen. After adding serial dilutions of perlecan, binding was detected with antibodies to perlecan, followed by a peroxidase-labelled second antibody and colorimetric reaction as in Fig. 6.

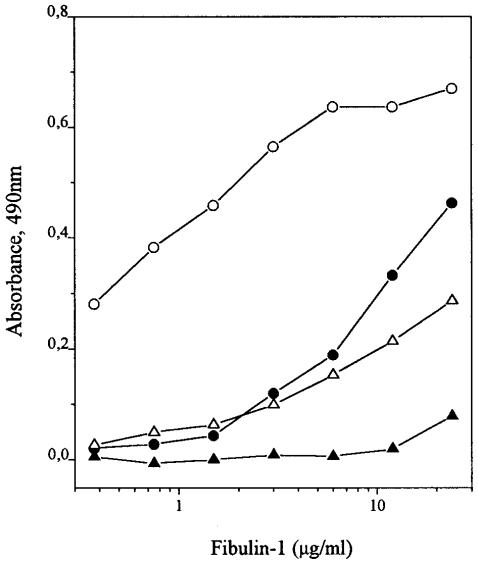

Fig. 9. Binding of human laminin isoforms to fibulin-1. Serial dilutions of fibulin-1 were added to wells coated with AmB1sB2e (○,●) or AmB1eB2e (△,▲) in the presence of either 2 mM $CaCl_2$ (open symbols) or 10 mM EDTA (filled symbols). Binding was detected with an antiserum against fibulin-1, followed by a peroxidase-labelled second antibody and colorimetric reaction as in Fig. 6.

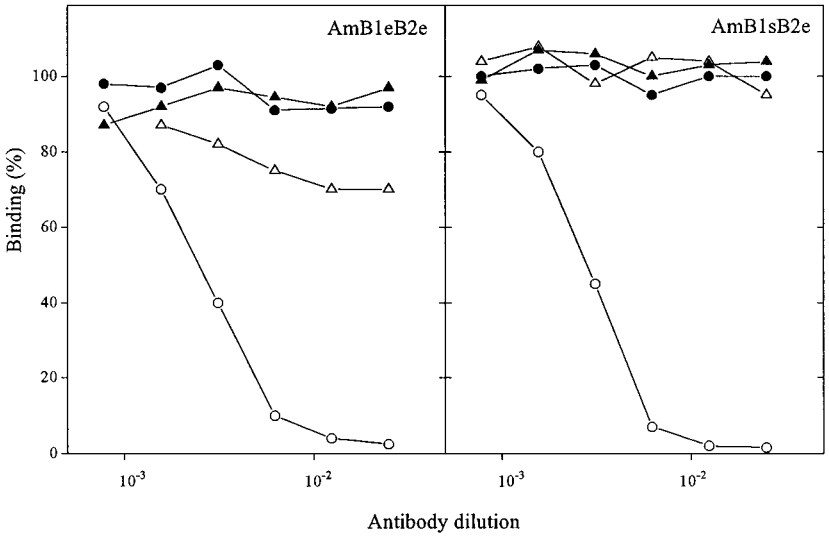

Fig. 10. Inhibition of HT 1080 cell adhesion to two laminin isoforms by monoclonal antibodies against integrin subunits. The antibodies used were culture medium containing anti-β_1 (○), and purified anti-β_3 (●), anti-α_2 (△) and anti-α_6 (▲) antibodies (stock concentration 200 μg/ml). In addition, no inhibition was observed with anti-α_3 and anti-α_5 (not shown). Non-inhibited binding (100%) corresponds to more than 50% of the cells bound (absorbance values 1.2-1.5; see Aumailley et al., 1989b).

Integrin-mediated cell adhesion activity

A mixture of the two human laminin isoforms was previously shown to mediate cell adhesion and spreading in a fashion comparable to EHS tumour laminin (Brown and Goodman, 1991). The two separated isoforms were now examined with HT 1080, HBL-100 and Rugli cell lines and found to have similar adhesive properties (not shown). The nature of cellular receptors involved was studied with HT 1080 cells and several inhibitory monoclonal antibodies to various integrin subunits (Fig. 10). Cell adhesion to both laminin isoforms could be completely blocked in a dose-dependent manner by antibodies to the β_1 subunit. A weak partial inhibition (~25%) was observed with antibodies to the α_2 subunit but only with the AmB1eB2e substrate. Antibodies against the subunits β_3, α_3, α_5 and α_6 had no or only small effects when used at similar concentrations. Adhesion of HT 1080 cells to EHS tumour laminin or its cell-adhesive fragment E8 was inhibited by both anti-β_1 and anti-α_6 antibodies, as shown previously (Aumailley et al., 1990). Adhesion of HT 1080 and HBL-100 cells to the two laminin isoforms was also not sensitive to synthetic GRGDS or RGES peptides up to a concentration of 500 μM, indicating that RGD-dependent integrins are not involved.

DISCUSSION

Our data demonstrate that neutral salt-extracted laminin from human placenta (Brown et al., 1990; Engvall et al., 1990) consists of two different isoforms with the chain compositions AmB1eB2e and AmB1sB2e. These isoforms were separated and their chains were identified by specific antibodies. Both laminins contained a non-covalently associated 67-80 kDa polypeptide, which probably represents the previously identified fragment merosin consisting of the G3-G5 domains from the C-terminal end of the Am chain (Ehrig et al., 1990). This explains the smaller size of the Am when compared to the Ae chain (Figs 2, 3) and agrees with a cross-shaped structure similar to that observed previously for EHS tumour laminin of the composition AeB1eB2e (Paulsson et al., 1987; Beck et al., 1990). On chromatography of the two placental isoforms on a Mono Q column, each eluted over a broad range of NaCl concentrations, with some overlap between the isoforms (Fig.1). Subtle differences in post-translational modifications, e.g. sialylation, may be responsible for this chromatographic heterogeneity. Differences in the sialic acid content of most of the more than 40 oligosaccharides have been demonstrated for EHS tumour laminin (Fujiwara et al., 1988) and it is likely that a similar heterogeneity may exist for the placental laminins.

The separation of the two placental isoforms supports previous notions that the B1e and B1s chains do not coexist in individual laminin molecules. This was previously indicated from antibody affinity chromatography of large pepsin fragments of placental laminins (Engvall et al., 1990), biosynthetic studies with muscle and glia-like cell lines (Green et al., 1992) and different localizations in muscle and renal basement membranes (Sanes et al., 1990). A lack of coexistence was also predicted from the analysis of the ionic interaction potentials within the coiled-coil region of laminin's long arm, which is considered to represent the site for the heterotrimeric chain assembly (Engel et al., 1991; Hunter et al., 1992; Beck et al., 1993). The assembled isoforms AmB1eB2e and AmB1sB2e can, however, be found in the same anatomical localizations such as in the perisinusoidal space of liver (Wewer et al., 1992) or the trophoblastic basement membranes (Engvall et al., 1990), the latter anatomical structure being the source of the two laminin isoforms described here.

A major aim of the study was to compare the binding potential of laminin isoforms, that are different from the mouse EHS tumour laminin. The two human placental laminins had a comparable affinity for nidogen, a ubiquitous 150 kDa basement membrane component (Timpl and Aumailley, 1993). The nidogen-binding site of EHS tumour laminin has been assigned to a single EGF-like motif within the short arm structure of the B2e chain (Gerl et al., 1991; Mayer et al., 1993). The same motif in the human B2e chain shows 97% sequence identity (Pikkarainen et al., 1988). The high similarity as well as inhibition data with the recombinant fragment B2III3-5 strongly indicate that the same motif of the human B2e chain is responsible for nidogen binding. The affinity of binding via this site is also apparently independent of the association with either Ae, Am, B1e or B1s chains.

The two placental laminin isoforms contained non-cova-

lently attached nidogen but in substoichiometric amounts (3-70%), as shown by radioimmunoassay. The association with some endogenous nidogen explains the 2- to 3-fold lower binding capacity when compared to AeB1eB2e laminin. The nidogen content of the latter laminin is >90% when extracted and purified from the EHS tumour (Paulsson et al., 1987; Mann et al., 1988) under similar conditions. This indicates that human nidogen is more sensitive to endogenous proteolysis than mouse nidogen (Dziadek et al., 1985), which was recently shown directly by comparing the two proteins in recombinant form (R. Nischt, U. Mayer and R. Timpl, unpublished data). A high nidogen content was also observed for Am chain-containing laminin from mouse heart (Paulsson and Saladin, 1989), showing that the association with a particular isoform is not correlated with a rapid degradation.

Nidogen binding to EHS tumour laminin has been recently shown to be important for the formation of ternary complexes with collagen IV and perlecan (Fox et al., 1991; Battaglia et al., 1992). This binding occurs via different globular domains of nidogen (Reinhardt et al., 1993) and is considered to be an essential step in connecting the networks of laminin and collagen IV within basement membranes (Aumailley et al., 1993). As shown here, nidogen may have a similar bridging function for other laminin isoforms as long as they share the B2e chain. This was demonstrated by the correlation between the nidogen content of the AmB1eB2e and AmB1sB2e isoforms and their ability to bind collagen IV and perlecan, as well as by enhancement of low binding through addition of exogenous nidogen. A low affinity of these laminins for collagen IV cannot, however, be excluded and will require studies with laminin from sources that lack nidogen entirely.

Two mechanisms of EHS tumour laminin binding to perlecan have been previously described (Battaglia et al., 1992). One operates by nidogen bridging of the B2e chain motif to a structure in the core protein of the proteoglycan. The same mechanism is probably responsible for the binding of isoforms AmB1eB2e and AmB1sB2e to perlecan. The second mechanism involves the binding of the heparan sulphate side-chains of perlecan to a C-terminal site in the Ae chain of EHS tumour laminin. This site is located in fragment E3 of laminin, which corresponds to the G4-G5 domains, and binding is inhibited by low concentrations of heparin (IC_{50}=0.8 μg/ml) or heparan sulphate (IC_{50}=5 μg/ml) (Battaglia et al., 1992). Binding of the placental laminin isoforms to perlecan was insensitive to heparin (IC_{50}>500 μg/ml), thus excluding this alternative binding mechanism. This could reflect a low sequence identity (~40%) between fragment E3 and the corresponding region in the Am chain (Ehrig et al., 1990). However, as shown here and previously (Engvall et al., 1992; Brown et al., 1993), Am chain-containing laminins show some partial binding in heparin affinity chromatography. This indicates a weak affinity, which may not be sufficient to bind to the heparan sulphate of EHS tumour perlecan.

The only striking difference observed between the placental isoforms AmB1eB2e and AmB1sB2e was the binding to fibulin-1 (BM-90). This calcium-binding 90 kDa protein was detected in several basement membranes and showed binding to EHS tumour laminin and nidogen, and to plasma fibronectin (Kluge et al., 1990; Balbona et al., 1992; Pan et al., 1993). These multiple interactions of fibulin-1 may allow it to function as a link molecule in basement membranes, in a similar way to nidogen. Its ability to interact with some laminin isoforms and not others may thus confer different structures and consequently different physicochemical or functional properties on basement membranes containing different laminin isoforms. A major, calcium-dependent fibulin-1 binding site could be localized to EHS laminin fragment E3 (Pan et al., 1993) but may not represent the only binding site. Since both placental isoforms share the Am chain but differ considerably in activity, it is unlikely that this chain is involved in binding rather than the B1s chain. This prediction needs to be confirmed by isolating a corresponding fragment possessing the binding site.

A few studies have demonstrated that intact laminin isoforms possessing Am chains promote cell adhesion and neurite outgrowth (Brown and Goodman, 1991; Engvall et al., 1992). Here we could show that adhesion is blocked by anti-β_1 integrin antibodies as was shown for neurite stimulation (Engvall et al., 1992). The nature of the α subunits involved could not be clarified but may not include α_2, α_3, α_5 and α_6. Previous studies with a large pepsin fragment of human placental laminin implied the participation of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins (Gehlsen et al., 1988; Sonnenberg et al., 1991) but the structure and chain composition of the fragments used have remained unclear. More recent studies with transfected cells having a very restricted integrin pattern showed binding of α6β1 to Am chain-containing laminins from heart and placenta, which was, however, not as strong as to EHS tumour laminin (Delwel et al., 1993). Similar data with transfectant cells were obtained for $\alpha_3\beta_1$, but here again the major ligand is apparently another laminin isoform, kalinin (A. Sonnenberg, personal communication). The actual integrins involved in the adhesion of cells with a complex integrin repertoire to the two placental laminins remain therefore to be established. Our present and previous antibody inhibition data (Brown and Goodman, 1991) indicate only a minor, if any, role for $\alpha_3\beta_1$ and $\alpha_6\beta_1$.

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