Identification of the B1 and B2 subunits of human placental laminin and rat parietal-yolk-sac laminin using antisera specific for murine laminin- β -galactosidase fusion proteins

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Antisera raised against fusion proteins consisting of murine laminin B1 and B2 subunit sequences fused to the C-terminus of *Escherichia coli* β -galactosidase were tested for their subunit specificity on Western blots of deglycosylated murine Engelbreth-Holm-Swarm (EHS) laminin. The antisera raised against B2 subunit sequences (anti-XLB2.1 and anti-XLB2.2) bound only to the EHS laminin B2 subunit. One of the antisera raised against B1 subunit sequences (anti-XLB1.2) was specific for the B1 subunit, whereas two others (anti-XLB1.1 and anti-XLB1.3) cross-reacted with the EHS laminin B2 subunit. Gold-labelled heparin-albumin was shown to bind specifically to the A subunit of deglycosylated EHS laminin on Western blots. These reagents were used to identify the homologous subunits in rat parietal-yolk-sac laminin and human placental laminin. The anti-(fusion protein) antisera identified the B1 and B2 subunits of the rat laminin, and these were similar in size to the murine EHS B subunits. Human placental laminin gave bands of 400, 340, 230, 190 and 180 kDa on reducing SDS/PAGE. The anti-(fusion protein) antisera identified the 230 and 190 kDa bands as the B1 and B2 subunits respectively. Gold-labelled heparin-albumin bound to the 400, 340 and 190 kDa bands of human placental laminin and so did not unambiguously identify a single A subunit. The human placental laminin may contain a mixture of isoforms, with alternative subunits substituting for the A subunit.

INTRODUCTION

Laminin, a large glycoprotein with a molecular mass of about 800 kDa, is a major component of basement membranes. It is composed of three glycosylated subunits, an A subunit of about 400 kDa and B1 and B2 subunits of about 200 kDa each (Timpl *et al.*, 1979). The three subunits are arranged to form a cross-shaped molecule, as seen by rotary shadowing (Engel *et al.*, 1981). A model for the structure of the laminin molecule has been proposed (Hogan *et al.*, 1985) in which each of the three subunits form one of the short arms of the cross and together extend down the long arm (Fig. 1). Laminin plays a major structural and functional role in the basement membrane, interacting with other basement-membrane components such as type IV collagen (Charonis *et al.*, 1986), heparan sulphate proteoglycan (Sakashita *et al.*, 1980) and nidogen (Paulsson *et al.*, 1987). The laminin molecule contains several cell-binding sites (Goodman *et al.*, 1987; Graf *et al.*, 1987; Grant *et al.*, 1989) and has been found to promote cell migration (McCarthy & Furcht, 1984; Öcalan *et al.*, 1988) and differentiation (Manthorpe *et al.*, 1983; Grant *et al.*, 1989).

The sequences of cDNAs for all three of the murine laminin subunits (Sasaki & Yamada, 1987; Sasaki *et al.*, 1987, 1988) and for the human laminin B1 and B2 subunits have been published (Pikkarainen *et al.*, 1987, 1988) and show laminin to be highly conserved between mouse and man, with sequence similarities in excess of 88% for the B subunits and 74% for a partial A subunit sequence (Olsen *et al.*, 1989) at the polypeptide level.

Much of the work to date on the structure and function of laminin has used murine laminin purified from the Engelbreth-Holm-Swarm (EHS) tumour, although recent studies suggest some structural differences between EHS laminin and laminin of non-neoplastic origin (for a review, see Beck *et*

al., 1990). Straightforward procedures have been established for the purification of laminin from the murine EHS tumour (Paulsson *et al.*, 1987) and from the rat parietal-yolk-sac (PYS) tumour (Engvall *et al.*, 1983). Methods for the isolation of human placental laminin have been described (Ohno *et al.*, 1983; Wewer *et al.*, 1983; Dixit, 1985), and laminin from this source gives a more complex pattern of bands on reducing SDS/PAGE than does murine EHS laminin (Dixit, 1985; Ohno *et al.*, 1986). In particular, placental laminin from a number of species appears to co-purify with an additional subunit, the M subunit, which has a molecular mass between that of the A and the B subunits (Ohno *et al.*, 1986). To date, no objective assessment of the identity of the bands seen in reducing SDS/polyacrylamide gels of human placental laminin has been reported. In the present study we identified the B subunits of human placental laminin using subunit-specific antisera raised against bacterial fusion proteins consisting of fragments from the B subunits of murine laminin fused to the C-terminus of *Escherichia coli* β -galactosidase.

MATERIALS AND METHODS

Reagents

Murine EHS laminin was obtained from either Gibco (Paisley, Renfrewshire, Scotland, U.K.) or Biogenesis (Bournemouth, Dorset, U.K.). Rat PYS laminin was from Biogenesis. Rabbit anti-(murine laminin) antiserum was obtained from Gibco. Ponceau S and peroxidase-conjugated sheep anti-(rabbit IgG) antiserum were from Sigma. Glycopeptidase F was purchased from Boehringer Mannheim or from Sigma. Silver enhancement reagents for detection of gold conjugates were obtained from Janssen (Beerse, Belgium). Phosphate-buffered saline (PBS) con-

Abbreviations used: EHS, Engelbreth-Holm-Swarm; PYS, parietal yolk sac; PBS, phosphate-buffered saline (composition and pH are given in the text); PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide.

tained NaCl (8 g/l), KCl (0.2 g/l), Na₂HPO₄ (1.15 g/l) and KH₂PO₄ (0.2 g/l), pH 7.2.

Purification of human placental laminin

The method used was based on that of Dixit (1985); all steps were carried out at 4 °C. The outer membrane and umbilical cord of full-term human placentae were removed, and the remaining tissue was homogenized in a Waring blender. After washing with cold tap water to remove the blood, the tissue was extracted for 60 h with 3 litres of solution I (0.02 M-Tris/1 M-NaCl/10 mM-EDTA/0.3 mM-PMSF/10 mM-NEM/10 mM-Na₃N₃/0.14 mM-*p*-hydroxymercuribenzoate, pH 7.4) containing 3 % (v/v) Triton X-100/kg. After centrifugation at 35000 *g* for 30 min, the NaCl concentration of the supernatant was adjusted to 5 M. Precipitated material was pelleted at 35000 *g* for 30 min, then redissolved in solution I. After centrifugation at 20000 *g* for 30 min, NaCl was added to the supernatant to 5 M. Precipitated proteins were pelleted as before, then redissolved in solution I containing 2 M-urea. The material was concentrated and dialysed against solution II (2 M-urea/0.02 M-Tris/0.3 mM-PMSF/10 mM-Na₃N₃, pH 7.4), containing 0.02 M-NaCl. The dialysed solution was clarified by centrifugation at 15000 *g* for 10 min. The supernatant was loaded on to a 2.5 cm × 50 cm column of Whatman DE-52 in solution II containing 0.02 M-NaCl. Column-bound material was eluted with solution II containing 0.5 M-NaCl, concentrated, and dialysed against 0.5 M-NaCl/0.05 M-Tris/0.3 mM-PMSF/10 mM-EDTA/10 mM-Na₃N₃, pH 7.4. This was passed through a 2.5 cm × 60 cm Sepharose CL-6B column using the same buffer. The first absorbance peak at 280 nm was collected, concentrated, dialysed against PBS containing 10 mM-Na₃N₃ and stored at 4 °C or -20 °C.

Fusion proteins

Fusion proteins XLB1.1, XLB1.2, XLB1.3, XLB2.1 and XLB2.2 were described previously (Brown *et al.*, 1989). Laminin-encoding plasmids pPE9, pPE49 and pPE386 (Barlow *et al.*, 1984) were obtained from Dr. Brigid Hogan, National Institute for Medical Research, Mill Hill, London N.W.7, U.K. Expression vectors pEX1, pEX2 and pEX3 (Stanley & Luzio, 1984) and *E. coli* pop2136 (F⁻, *endA*, *thi*, *hsdR*, *malT*, P⁻, *CI857*, *malPQ*; derived by Dr. O. Raibaud, Institut Pasteur, Paris, France) were kindly provided by Dr. John Doorbar, Department of Cancer Studies, University of Birmingham, Birmingham, U.K. Cloning techniques were as described by Maniatis *et al.* (1982). A 50 ng portion of gel-purified laminin DNA fragment was ligated to 25 ng of linearized and phosphatase-treated vector. The ligation mixtures were used to transform competent *E. coli* pop2136 as described by Hanahan (1983), except that the heat shock was at 32 °C and subsequent incubation was at 30 °C for 40 h. Recombinants with in-frame inserts in the correct orientation were selected by preparing small-scale protein preparations (Doorbar *et al.*, 1986) and looking for a protein of increased molecular mass relative to β -galactosidase. Fusion proteins were purified in bulk as outlined by Doorbar *et al.* (1986). Anti-(fusion protein) antisera were prepared by immunizing New Zealand White rabbits (Porcellus, Heathfield, East Sussex, U.K.) and Dutch-Lop crossed rabbits (Froxfield, Petersfield, Hants., U.K.) at three subcutaneous sites with 200 μ g of the fusion proteins per immunization emulsified in complete Freund's adjuvant for the first immunizations and incomplete Freund's adjuvant for subsequent immunizations.

Deglycosylation of laminin

Laminin (1 mg/ml) containing 0.4 % (w/v) SDS was heated for 3 min in a boiling-water bath. Laminin (500 μ g/ml) was digested overnight at 37 °C with glycopeptidase F in 0.064 M-

sodium phosphate buffer, pH 7.1, containing 0.5 % (v/v) Triton X-100. Glycopeptidase F (6 or 60 units/ml) was used for complete deglycosylation, depending on the batch of enzyme. A unit of enzyme activity is that which hydrolyses 1 nmol of dansyl-fetuin glycopeptide in 1 min at 37 °C at pH 7.2.

SDS/PAGE

For reducing SDS/PAGE, samples were mixed with an equal volume of sample buffer [80 mM-Tris/2 % (w/v) SDS/10 % (v/v) glycerol/0.1 % (w/v) Bromophenol Blue/5 % (v/v) 2-mercaptoethanol, pH 6.8] and heated for 5 min in a boiling-water bath. For non-reducing SDS/PAGE, the 2-mercaptoethanol was omitted from the sample buffer. Samples were electrophoresed on SDS/polyacrylamide gels as described by Laemmli (1970).

Western blotting

Laminin or deglycosylated laminin (3 μ g/track) was run on an SDS/6.5 %-(w/v)-polyacrylamide gel (Laemmli, 1970) and electroblotted on to nitrocellulose (Towbin *et al.*, 1979). Filters were stained with a 1:10 dilution of Ponceau S in water for 10 min, rinsed with distilled water and the positions of the stained bands were marked with a pencil. For a permanent protein stain, filters were incubated in 0.1 % (w/v) Amido Black in methanol/acetic acid/water (45:7:48, by vol.) for 10 min, then destained with methanol/acetic acid/water (45:7:48, by vol.). Filters to be probed with antisera were blocked by incubating with PBS containing 3 % (w/v) BSA and 0.05 % (v/v) Tween-20 for 1 h, which removed the reversible Ponceau S stain. Rabbit antisera and peroxidase-conjugated sheep anti-(rabbit IgG) antiserum (1:1000) were diluted in blocking buffer. Filters were washed with 0.1 % (v/v) Tween-20 in PBS. The bound peroxidase was detected with 0.05 % (w/v) diaminobenzidine and 0.03 % (v/v) H₂O₂ in PBS.

Probing nitrocellulose filters with gold-labelled heparin-albumin

After electroblotting, staining with Ponceau S and blocking as described above, nitrocellulose filters were incubated with a 1:50 dilution of heparin-albumin gold (20 nm particle size) (Sigma) for approx. 6 h. For silver enhancement, IntenSE BL enhancer and initiator (Janssen) were used according to the manufacturer's instructions.

RESULTS

Fusion proteins

A series of expression plasmids encoding β -galactosidase-laminin fusion proteins were constructed. The fusion proteins consisted of murine laminin B1 and B2 subunit sequences fused to the C-terminus of *E. coli* β -galactosidase. On induction, all of the recombinants were found to express hybrid proteins of the expected size (Fig. 1). Rabbit polyclonal antisera raised against these fusion proteins have been shown to bind to murine EHS laminin in an e.l.i.s.a. (Brown *et al.*, 1989). A series of experiments was carried out to characterize their specificities for individual laminin subunits using deglycosylated murine EHS laminin.

Deglycosylation of murine EHS laminin

On SDS/polyacrylamide gels the B1 and B2 subunits of murine EHS laminin run together (see Fig. 2, lane a, and Timpl *et al.*, 1979). To attempt to separate the two B subunits by SDS/PAGE, laminin was deglycosylated before electrophoresis with glycopeptidase F, which cleaves the *N*-glycan linkage of glycoproteins between asparagine and the carbohydrate chain (Tarentino *et al.*, 1985).

After deglycosylation, the reduced laminin resolved into three

Fig. 1. Model of laminin structure showing positions of fusion-protein sequences

The model of the laminin structure proposed by Hogan *et al.* (1985), with subunit domains as described by Sasaki & Yamada (1987) and Sasaki *et al.* (1987, 1988). ○, globular domains; □, cysteine-rich domains; ∞, α -helical domains; $\triangle$, interruption to α -helix. The bars show the location of the sequences contained within the fusion proteins XLB1.1, XLB1.2, XLB1.3, XLB2.1 and XLB2.2. Numbers show the amino acids of the B1 and B2 subunits represented in the fusion proteins.

major bands on SDS/polyacrylamide gels rather than two, as shown in Fig. 2, lane b. The position of the band of the highest molecular mass was consistent with this being the deglycosylated A subunit, and the two bands of lower molecular mass ran in positions consistent with them representing the deglycosylated B1 and B2 subunits. From the published DNA sequences, the deglycosylated murine B1 subunit (predicted molecular mass 196 903 Da; Sasaki *et al.*, 1987) would be expected to run at a slightly higher position on the gel than the deglycosylated murine B2 subunit (predicted molecular mass 177 541 Da; Sasaki & Yamada, 1987).

Specificity of anti-(fusion protein) antisera for murine EHS laminin subunits

The results of probing nitrocellulose filters, on to which glycosylated and deglycosylated murine EHS laminin had been transferred, with rabbit antisera raised against the fusion proteins are shown in Fig. 3(a). All the anti-(fusion protein) antisera bound to a broad band of approx. 200 kDa on glycosylated laminin. The antisera raised against the two B2 subunit fusion proteins (anti-XLB2.1 and anti-XLB2.2) bound to only the

Fig. 2. Reducing SDS/6%-(w/v)-PAGE of glycosylated and deglycosylated murine EHS laminin

A 5 μ g portion of murine EHS laminin (Biogenesis) was loaded per track. Lane a, glycosylated laminin. Arrows A and B point to the A and B subunits respectively. The faint band of lower molecular mass (arrow N) is probably nidogen, and is not seen with murine EHS laminin obtained from Gibco. Lane b, laminin deglycosylated with 0.6 unit of glycopeptidase F (Boehringer Mannheim).

Fig. 3. Binding of rabbit anti-(fusion protein) antisera to glycosylated and deglycosylated murine EHS laminin

Murine EHS laminin (Gibco) (3 μ g/track) was run on a reducing SDS/6%-(w/v)-polyacrylamide gel either glycosylated (G) or deglycosylated with 0.036 unit of glycopeptidase F (Boehringer Mannheim) (D). The protein bands were transferred to nitrocellulose. (a) Filters probed with rabbit anti-(fusion protein) antisera: a, anti-XLB1.1 (1:25000 dilution); b, anti-XLB1.2 (1:25000 dilution); c, anti-XLB1.3 (1:50000 dilution); d, anti-XLB2.1 (1:100000 dilution); e, anti-XLB2.2 (1:100000 dilution). (b) Filter probed with gold-labelled heparin-albumin (1:50 dilution). Lines at the right-hand side of each filter indicate the positions of the Ponceau S-stained deglycosylated bands. Positions of Gibco prestained protein standards are shown at the left on the Figure.

lower of the two resolved B subunits (lanes d and e). The anti-(B1 subunit) antisera (anti-XLB1.1, anti-XLB1.2 and anti-XLB1.3) bound predominantly to the higher B subunit, although the anti-XLB1.1 and anti-XLB1.3 antisera (lanes a and c) also showed some binding to the lower-molecular-mass band.

In addition, all the anti-(fusion protein) antisera showed a low level of binding to a band which migrated with a molecular mass greater than the 400 kDa subunit (see Fig. 3). This band was not visible on Coomassie Blue-stained gels (Fig. 2, lane b), so represents, quantitatively, a minor component. To confirm that the major protein band at 400 kDa had been correctly identified as the laminin A subunit, electroblots of deglycosylated murine EHS laminin were probed with gold-labelled heparin-albumin. Heparin has been shown to bind to the A subunit, but not the B subunits, of reduced and alkylated EHS laminin (Arumugham *et al.*, 1988). As the results in Fig. 3(b) show, the labelled heparin bound to the major 400 kDa band, thus confirming its identity as the A subunit. The heparin-gold also bound to a band of slightly lower molecular mass than the A subunit. We propose that the high-molecular-mass band recognized by the anti-(B subunit fusion protein) antisera represents a B1B2 dimer. Its position on the gel relative to the A subunit is consistent with the predicted molecular masses of a deglycosylated B1B2 dimer and a deglycosylated A subunit, which are 374 444 Da and 335 729 Da (Sasaki *et al.*, 1988) respectively.

These experiments demonstrate that the anti-(B subunit fusion protein) antisera and the heparin-gold can be used as specific probes for the three subunits of murine EHS laminin. Using these probes we examined the identity of the bands obtained in SDS/PAGE of rat PYS laminin and human placental laminin.

Comparison of murine EHS laminin, rat PYS laminin and human placental laminin by SDS/PAGE

Human laminin was isolated from placenta, with yields of approx. 20 mg/kg, and compared with murine EHS laminin and rat PYS laminin on SDS/polyacrylamide gels (see Fig. 4). Under non-reducing conditions, all three types of laminin migrated as a single broad band near the top of the gel. When reduced, murine EHS laminin and rat PYS laminin appeared very similar, each giving two bands of approx. 400 and 200 kDa. Human placental laminin gave a more complex pattern after reduction: bands with estimated molecular masses of 340, 230, 190 and 180 kDa were evident, and a faint band of about the same molecular mass as the rodent laminin A subunits was also seen. Bands of lower molecular mass were also observed, which we believe represent breakdown products that appear on storage.

Fig. 4. SDS/PAGE of murine EHS, rat PYS and human placental laminin

Reducing (a) and non-reducing (b) SDS/6.5%-(w/v)-polyacrylamide gels of: a, human placental laminin; b, rat PYS laminin; c, murine EHS laminin (Gibco) with 5 µg of protein per track. The positions of the Gibco prestained protein standards are shown at the right-hand side of each gel. The human-placental-laminin bands referred to in the text are indicated by arrows.

Characterization of rat PYS laminin using subunit-specific antisera

After deglycosylation with glycopeptidase F, rat PYS laminin resolved into three bands on reducing SDS/PAGE with mobilities identical with those of the subunits of deglycosylated murine EHS laminin (results not shown). Both the glycosylated and deglycosylated forms of rat PYS laminin were probed with the anti-(fusion protein) antisera (Fig. 5). The anti-(B1 subunit fusion protein) antisera bound to the higher-molecular-mass B subunit and the anti-(B2 subunit fusion protein) antisera bound to the lower-molecular-mass B subunit of deglycosylated rat laminin. Thus rat PYS laminin appears to have a very similar subunit composition to murine EHS laminin. However, in contrast with murine EHS laminin, no cross-reactivity of anti-XLB1.1 or anti-XLB1.3 with the B2 subunit of rat PYS laminin was observed.

Characterization of human placental laminin using subunit-specific probes

We used the antisera raised against the murine B subunit fusion proteins in Western blots to identify the bands representing the human B1 and B2 subunits (Fig. 6). All three anti-(B1 subunit fusion protein) antisera bound to the 230 kDa band, identifying this as the B1 subunit. Anti-XLB1.1 antiserum also reacted weakly with the 190 kDa and the 340 kDa bands (lane a). The anti-(B2 subunit fusion protein) antisera bound predominantly to the 190 kDa band, identifying this as the B2 subunit. A faint band with a molecular mass lower than 180 kDa was also labelled, and probably represents a breakdown product of the B2 subunit. They also bound to a band with a slightly higher molecular mass than the 400 kDa band, which was not

Fig. 5. Binding of rabbit anti-(fusion protein) antisera to glycosylated and deglycosylated rat PYS laminin

Rat PYS laminin (3 µg/track), either glycosylated (G) or deglycosylated with 0.6 unit of glycopeptidase F (Sigma) (D), were run on an SDS/6%-(w/v)-polyacrylamide gel and electroblotted on to nitrocellulose. Filters were stained with Amido Black (a) or probed with rabbit anti-(fusion protein) antisera: b, anti-XLB1.1 (1:25000 dilution); c, anti-XLB1.2 (1:25000 dilution); d, anti-XLB1.3 (1:50000 dilution); e, anti-XLB2.1 (1:100000 dilution); f, anti-XLB2.2 (1:100000 dilution). Lines at the right-hand side of each filter indicate the positions of the Ponceau S-stained deglycosylated bands. Positions of Gibco prestained protein standards are shown at the left of the Figure.

Fig. 6. Binding of rabbit anti-(fusion protein) antisera to human placental laminin

Human placental laminin (5 μ g/track) was run on an SDS/6.5%-(w/v)-polyacrylamide gel, electroblotted on to nitrocellulose and probed with: a, rabbit anti-XLB1.1 (1:1000 dilution); b, rabbit anti-XLB1.2 (1:2000 dilution); c, rabbit anti-XLB1.3 (1:1000 dilution); d, rabbit anti-XLB2.1 (1:20000 dilution); e, rabbit anti-XLB2.2 (1:20000 dilution); f, rabbit anti-(murine EHS laminin) (1:5000 dilution); g, gold-labelled heparin-albumin (1:50 dilution). Lines at the right-hand side of each filter indicate the positions of the major Ponceau S-stained bands. On these blots, the 230 kDa band was not visible with Ponceau S. Positions of Gibco prestained protein standards are shown at the left of the Figure.

visible on Coomassie Blue-stained gels. Anti-XLB2.2 also showed some binding to the 340 kDa subunit (Fig. 6, lane e). None of the anti-(fusion protein) antisera bound to the 180 kDa subunit. In contrast with murine laminin, the B subunits of human placental laminin are resolved on SDS/polyacrylamide gels in their native, glycosylated, forms. Additional evidence for the identification of the B subunits of human placental laminin was obtained by probing Western blots with a rabbit antiserum raised against intact murine EHS laminin (Fig. 6, lane f). This antiserum bound only to the 230 kDa and 190 kDa bands of human placental laminin and to a doublet in the region of the 400 kDa subunit. Western blots of human placental laminin were also probed with gold-labelled heparin-albumin to attempt to identify the A subunit. As shown in Fig. 6, lane g, gold-labelled heparin-albumin bound predominantly to bands with molecular masses of 400, 340 and 190 kDa and did not identify a single band as the A subunit.

Search for sequence similarity

Since the anti-XLB1.1 and anti-XLB1.3 antisera appeared to cross-react with the B2 subunit of murine EHS laminin, the Intelligenetics (Mountain View, CA, U.S.A.) PEP computer program was used to search for sequence similarity between the laminin sequences encoded within the three B1 subunit fusion proteins (amino acids 1340–1834) and the whole of the B2 subunit. A search for a 100% match of at least six amino acids found two such sequences, namely TCGTPP, which was in the part of the B1 subunit contained within XLB1.2, and EEAKRA, which was in the region contained within XLB1.1. An antipathicity plot using the Hopp and Woods values on the Intelligenetics PEP program indicated that the sequence TCGTPP resided in a very hydrophobic part of the B1 sequence and was therefore unlikely to be antigenic. The sequence

EEAKRA was in a very hydrophilic region of the B1 subunit and this sequence may, at least in part, account for the observed cross-reactivity of anti-XLB1.1 with the B2 subunit of murine EHS laminin. No sequence identity was found between the laminin B2 sequences encoded within XLB2.1 and XLB2.2 and the B1 subunits.

DISCUSSION

Immunochemical studies of laminin carried out so far have mainly used domain-specific antisera. An alternative approach which we have adopted is to use antisera specific for the individual laminin subunits.

Subunit-specific anti-(laminin fusion protein) antisera similar to those described here have been produced by Wu *et al.* (1988). They constructed a B1 subunit fusion protein, F3, containing the C-terminal 944 amino acids of the B1 subunit of murine laminin and a B2 subunit fusion protein, E4, containing a rat laminin sequence analogous to the murine sequence contained in XLB2.2. In agreement with Wu *et al.* (1988), we have found that the anti-(B2 subunit fusion protein) antisera are subunit-specific in their binding to murine laminin, and that two of the anti-(B1 subunit) antisera bind to two bands in Western blots of deglycosylated murine laminin. One of these bands is identical in molecular mass with the band labelled by the anti-(B2 subunit fusion protein) antisera, and may represent a cross-reaction with the B2 subunit. The hydrophilic sequence EEAKRA (amino acids 1555–1560 of the murine laminin B1 subunit), which also occurs in the B2 subunit, may, at least in part, account for this cross-reactivity. This hypothesis can be tested using the synthetic peptide to inhibit binding. The anti-XLB1.2 antiserum does not show this cross-reactivity and is a laminin-B1-subunit-specific probe.

We have used gold-labelled heparin-albumin as a specific probe for the laminin A subunit on Western blots and have shown that the heparin-binding site on the laminin A subunit must reside on the polypeptide backbone and not on carbohydrate side chains, since the probe bound to the deglycosylated A subunit. Several heparin-binding sites have been identified on EHS laminin (Ott *et al.*, 1982; Skubitz *et al.*, 1988) and localized to the A subunit (Arumugham *et al.*, 1988) and to the B1 subunit (Charonis *et al.*, 1988). Our results suggest, in agreement with Arumugham *et al.* (1988), that only the heparin-binding domain on the A subunit is detectable after the reduction and denaturation of laminin.

The heparin binding to murine EHS laminin on Western blots indicated the presence of an A subunit doublet. An A doublet has been observed previously in laminin-secreting cell lines (Peters *et al.*, 1985; Wu *et al.*, 1988), and Peters *et al.* (1985) have suggested that the truncated form of the A subunit may be necessary for the secretion of laminin. The truncated A subunit observed here in the EHS laminin must be a minor component, since it was not detected on gels stained with Coomassie Blue (Fig. 2).

The procedure for the purification of human placental laminin described by Dixit (1985), on which our method was based, yielded a protein which appeared on reducing SDS/polyacrylamide gels as bands of 350–370, 230, 190 and 180 kDa. These were designated as the A, M, B1 and B2 subunits respectively. Although precise molecular-mass determinations are difficult at these high molecular masses, the positions of bands seen in this study at 230, 190 and 180 kDa are clear with respect to the 200 kDa-molecular-mass marker, the myosin heavy chain, and appear to correspond exactly to the three lower-molecular-mass bands seen by Dixit (1985). However, our data demonstrate that the 230 kDa subunit, which was designated

'M' by Dixit, is the B1 subunit, and the 190 kDa subunit, designated 'B1' by Dixit, is the B2 subunit. The identity of the 180 kDa band, which was designated 'B2' by Dixit, remains to be established. Since this 180 kDa band co-purified with laminin and remained complexed with it when it was electrophoresed under non-reducing conditions, it must be either a laminin subunit or a protein which is strongly complexed to the laminin molecule. Our preliminary data suggest that it is not antigenically related to either fibronectin or type IV collagen. In addition, we do not believe that it represents the human equivalent of nidogen, since murine EHS nidogen does not remain complexed with laminin when electrophoresed under non-reducing conditions.

It is not clear whether the 340 or 400 kDa band on our gels of human placental laminin corresponds to Dixit's 350–370 kDa band. One interpretation is that the 400 kDa band represents the human placental laminin equivalent of the murine EHS laminin A subunit, and the 340 and 190 kDa bands, which were also heparin-binding, may represent degradation products of this subunit. In this case the 190 kDa band observed on the gel would be a mixture of the B2 subunit and an A subunit degradation product, thereby accounting for its greater intensity in Coomassie Blue-stained gels than the B1 subunit (Fig. 4). A human placental laminin band migrating in SDS/PAGE with a molecular mass between that of the A and B subunits, the M subunit, was first described by Ohno *et al.* (1983) and they, too, suggested that this subunit may be a breakdown product of the A subunit (Ohno *et al.*, 1986). They showed that it occurred in placental tissue, but not in tumour-derived laminin, from a number of species including mouse and man.

An alternative interpretation for the identity of the 340 and 400 kDa bands is that they represent two unrelated subunits of laminin, and there is increasing evidence that alternative A subunits may exist. Paulsson & Saladin (1989) have identified a 300 kDa subunit in mouse heart laminin in addition to the A and B subunits, and a number of cell lines have been shown to secrete isoforms of laminin with either a truncated, or no, A subunit (Aratani & Kitagawa, 1988; Edgar *et al.*, 1988; Liesi & Risteli, 1989). Interestingly, some of these laminin variants contain a 180 kDa subunit, which may correspond to our lowest-molecular-mass human placental laminin subunit. Thus our human laminin preparation may contain a mixture of laminin isoforms with alternative A subunits. Since the A subunit has been shown to be involved in cell adhesion and differentiation (Ekblom, 1989; Grant *et al.*, 1989), these isoforms may confer functional heterogeneity on different basement membranes within the placenta.

We are very grateful to Mr. Terry Madigan, Ms. Sally Hatton and Mr. Neal Mathias for technical assistance and to Dr. Martin Mackay for his comments on the manuscript.

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