Monoclonal antibodies specific for the C-terminus of the laminin B2 subunit

Evidence for glycosylation differences between murine and human laminin

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We have raised a panel of monoclonal antibodies against a β -galactosidase fusion protein (XLB2.1) containing the C-terminal 153 amino acids of the murine laminin B2 subunit. Five of the nine antibodies characterized recognize human placental laminin as well as murine Engelbreth-Holm-Swarm (EHS)-tumour laminin. Only two of the antibodies recognize both rat parietal-yolk-sac laminin and murine EHS-tumour laminin. Two antibodies recognize an epitope on the human laminin B2 subunit which is masked by *N*-linked oligosaccharide in murine EHS-tumour laminin. These antibodies also fail to bind to laminin from adult-mouse tissues. These results demonstrate a species-specific difference in the glycosylation of the laminin B2 subunit.

INTRODUCTION

Laminin is a ubiquitous component of basement membranes that has been characterized biochemically using laminin purified from the murine EHS tumour. Three subunits, an A subunit of about 440 kDa and a B1 and B2 subunit, each of about 220 kDa (Timpl *et al.*, 1979), together form a cross-shaped molecule which can be revealed by electron microscopy using rotary-shadowing techniques (Engel *et al.*, 1981).

Laminin is known to be a highly conserved molecule, with an amino acid sequence similarity of 88% or greater for the B subunits and 76% for the A subunit of human and murine laminin (Pikkarainen *et al.*, 1987, 1988; Olsen *et al.*, 1989; Nissinen *et al.*, 1990). *Drosophila* DNA sequences almost identical with, and with domain structures similar to, those of the mammalian B1 and B2 subunits have also been published (Montell & Goodman, 1988; Chi & Hui, 1988). However, in spite of this high level of sequence identity, species-specific anti-laminin monoclonal antibodies have been described (Chung *et al.*, 1983), as well as others showing different binding patterns on murine and human tissues (Horikoshi *et al.*, 1988). In these cases the molecular basis of the inter-species differences in antibody recognition has not been determined.

Here we describe a series of monoclonal antibodies raised against a β -galactosidase fusion protein (XLB2.1) containing the C-terminal 153 amino acids of the murine laminin B2 subunit (Brown *et al.*, 1989, 1990). We have used these antibodies to demonstrate a difference in the post-translational modification of this region of the B2 subunit in murine and human laminin.

MATERIALS AND METHODS

Reagents

Murine EHS-tumour laminin was either obtained from Sigma (Poole, Dorset, U.K.) or purified as described by Paulsson *et al.* (1987). Rat parietal-yolk-sac (PYS) laminin was from Biogenesis (Bournemouth, Dorset, U.K.). Human placental laminin was purified as described previously (Brown *et al.*, 1990). The fusion

protein (XLB2.1) was described in Brown *et al.* (1989). Phosphate-buffered saline (PBS) contained NaCl (8 g/l), KCl (0.2 g/l) and Na_2HPO_4 (1.15 g/l), pH 7.2.

Monoclonal antibodies

CBA $\times$ Balb/c F_1 hybrid mice were immunized intraperitoneally at 3–4-week intervals with 25 μg or 50 μg of XLB2.1 emulsified in complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent immunizations. Mice were given a final tail-vein injection of 50 μg of XLB2.1 in PBS 3 days before fusing the splenocytes with the mouse myeloma cell line NSO, as described by Oi & Herzenberg (1980). The cells were grown and cloned by limiting dilution in Dulbecco's Modified Eagle's Medium containing 20% (v/v) foetal-calf serum (Sera-Lab, Crawley, Sussex, U.K.), hypoxanthine/aminopterin/thymidine (HAT) medium supplement (Sigma), 100 units of penicillin (Gibco, Paisley, Renfrewshire, Scotland, U.K.)/ml and 100 μg of streptomycin (Gibco)/ml. The heavy-chain subclass of the monoclonal antibodies was determined with a subtyping kit purchased from Serotec (Kidlington, Oxford, U.K.).

E.I.i.s.a.

E.I.i.s.a.s were based on the method described by Pfaff *et al.* (1982). Briefly, microtitre plates (Gibco) were coated with a 5 $\mu\text{g}/\text{ml}$ solution of antigen in 0.1 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 9.5. Bovine gelatin (0.5% in PBS) was used as blocking buffer and as dilution buffer for antibodies. PBS containing 0.05% (v/v) Tween-20 and 1% (v/v) butan-2-ol was used to wash plates between incubations. The binding of antibodies from hybridoma-tissue-culture supernatants was detected with a peroxidase-conjugated sheep anti-(mouse IgG) antiserum (Sigma) diluted 1:1000. 3,3',5,5'-Tetramethylbenzidine (100 $\mu\text{g}/\text{ml}$) and 0.0022% (w/v) H_2O_2 in 0.1 M-sodium acetate adjusted to pH 6 with citric acid was used as substrate/buffer; 2.5 M- H_2SO_4 was used to stop the reaction after 15 min at room temperature, and the A_{450} was read by using a Dynatech MR-600 microplate reader.

Abbreviations used: EHS, Engelbreth-Holm-Swarm; HAT, hypoxanthine/aminopterin/thymidine; PYS, parietal yolk sac; PBS, phosphate-buffered saline (for composition, see the text).

SDS/PAGE and Western blotting

SDS/PAGE and Western blotting were carried out as described previously (Brown *et al.*, 1990), except that monoclonal-antibody binding was detected with peroxidase-conjugated sheep anti-(mouse IgG) antiserum (Sigma).

Preparation of deglycosylated and denatured-reduced laminin

Deglycosylation of laminin was carried out as described by Brown *et al.* (1990). To prepare denatured-reduced laminin, laminin (500 µg/ml) was heated in a boiling-water bath for 5 min in the presence of 2% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and then incubated at room temperature for 1 h with 1/5 vol. of 0.75 M-iodoacetamide in 0.5 M-Tris (pH 8). The laminin was dialysed extensively against PBS, then stored in aliquots at -20 °C.

Extraction of laminin from murine tissues

Mouse tissues were weighed, minced, and washed on a sintered-glass funnel with cold distilled water, then mixed with SDS/PAGE reducing sample buffer (5 ml/g of tissue). After extensive mixing on a Whirlimixer, the samples were heated for 5 min in a boiling-water bath, then insoluble material was removed by centrifugation in an Eppendorf Microfuge (13 800 g for 5 min). The supernatants were stored in aliquots at -20 °C.

RESULTS

Monoclonal antibodies

Two fusions which used splenocytes from mice immunized with fusion protein XLB2.1 were carried out. Hybridoma supernatants from the first fusion were screened by e.l.i.s.a. for antibodies which bound to XLB2.1 but not to β-galactosidase. This yielded three monoclonal antibodies: 4.5D, 3.6B and 5.6D. In the second fusion, six monoclonal antibodies, 1.F5, 2.D12, 2.E1, 3.C12, 4.D11 and 6.H10, which bound to denatured-reduced murine EHS-tumour laminin (on the basis of e.l.i.s.a.), were selected for further study. The heavy-chain isotypes of these nine antibodies are given in Table 1.

Monoclonal-antibody binding to laminin from different species

Since the monoclonal antibodies were obtained by immunizing mice with a murine laminin sequence, antibodies which recognized native murine laminin were not expected. The antibodies were therefore characterized on laminin that had been denatured and reduced with SDS and 2-mercaptoethanol. Only one antibody, namely 6.H10, showed any significant binding to laminin that had not been so treated and at a much lower level than to denatured-reduced laminin (results not shown).

The binding of the monoclonal antibodies to denatured-reduced laminin from three different species was investigated by e.l.i.s.a. (Fig. 1). The six monoclonal antibodies from the second fusion, which were selected as binding denatured-reduced murine EHS-tumour laminin showed various patterns of cross-reactivity with human and rat laminin. All three of the antibodies from the first fusion bound to denatured-reduced human placental laminin, but none recognized rat PYS laminin, and only one, namely 4.5D, bound to denatured-reduced murine EHS-tumour laminin. The specificity of 3.6B and 5.6D was surprising, since the cDNA sequence used to construct the plasmid encoding fusion protein XLB2.1 was derived from murine cells.

Binding of monoclonal antibodies to mouse tissue extracts

To determine whether the unexpected pattern of recognition shown by 3.6B and 5.6D was due to a difference between the species or between the tissues from which the laminin originated, the binding of the monoclonal antibodies to a range of murine

tissues was tested. Western blots of crude extracts were probed with monoclonal antibodies 4.5D, 3.6B and 5.6D (Fig. 2). Monoclonal antibodies 3.6B and 5.6D bound to the B2 subunit

Table 1. Isotypes of anti-XLB2.1 monoclonal antibodies

Fusion	Monoclonal antibody	Isotype
1	4.5D	γ2a
	3.6B	γ1
	5.6D	γ1
2	1.F5	γ1
	2.D12	γ1
	2.E1	γ1
	3.C12	γ1
	4.D11	γ2a
	6.H10	γ1

Fig. 1. Binding of monoclonal antibodies to laminin from different species

The Figure shows maximum binding levels of anti-(fusion protein XLB2.1) monoclonal antibodies to denatured-reduced murine EHS-tumour laminin (■), rat PYS laminin (▒) and human placental laminin (□) measured by e.l.i.s.a.

Fig. 2. Binding of monoclonal antibodies 4.5D, 3.6B and 5.6D to murine tissue extracts

Electroblots of purified murine EHS-tumour laminin (E) (1 µg/track), SDS/2-mercaptoethanol extracts of murine liver (Li), brain (B), kidney (K), skin (Sk), heart (H), lung (L), skeletal muscle (M), spleen (S), placenta (P), and purified human placental laminin (HP) (1 µg/track), from SDS/6.5%-polyacrylamide gels were probed with 1:50 dilutions of hybridoma tissue-culture supernatants. Between 50 and 130 µg of the tissue extracts were run per track to give approximately equal protein loading over the molecular-mass range separated.

Fig. 3. Binding of monoclonal antibodies 4.5D, 3.6B and 5.6D to glycosylated (G) and deglycosylated (D) murine EHS-tumour laminin (Mu) and human placental laminin (Hu)

Deglycosylation was carried out as described by Brown *et al.* (1990). Samples (3 µg/track) were separated on SDS/6.5%-polyacrylamide gels and transferred to nitrocellulose filters. (a) Filter stained for total protein with Amido Black. (b) Filters probed with 1:50 dilutions of hybridoma-tissue-culture supernatants.

of human placental laminin, but not to EHS-tumour laminin or laminin in any of the tissue extracts, including the murine placental extract. By contrast, 4.5D bound to a band migrating as expected for the B2 subunit in kidney, heart, lung and placenta. This suggests that monoclonal antibodies 3.6B and 5.6D recognize epitopes on the B2 subunit which differ between mouse and human, rather than between placental and tumour laminins. The failure of 4.5D to bind to extracts of liver, brain, skin, skeletal muscle and spleen may indicate quantitative differences in the levels of laminin extracted from different tissues, rather than the absence of laminin B2 chains, since polyclonal rabbit antisera against either XLB2.1 or murine EHS-tumour laminin detect bands of approx. 200 kDa in every tissue extract (results not shown).

The six monoclonal antibodies from the second fusion all bound to the B2 subunit of murine EHS-tumour laminin and murine placental laminin in Western blots (results not shown).

Binding of monoclonal antibodies of deglycosylated laminin

The monoclonal antibodies described here were raised against a β -galactosidase fusion protein expressed in *Escherichia coli* in which the post-translational modifications characteristic of eukaryotes will not have occurred. Therefore the epitopes recognized by 3.6B and 5.6D in XLB2.1 may be masked in the murine, but not in the human, laminin B2 subunit, for example, by carbohydrate side chains. The ability of monoclonal antibodies 3.6B and 5.6D to bind to deglycosylated murine EHS-tumour laminin was therefore tested.

Murine EHS-tumour laminin and human placental laminin were deglycosylated with glycopeptidase F and probed on electroblots with monoclonal antibodies 3.6B, 4.5D and 5.6D (Fig. 3). Monoclonal antibody 4.5D bound to the B2 subunits of both glycosylated and deglycosylated murine and human laminin and showed that the glycopeptidase F had caused a marked reduction in the molecular masses of the B2 subunits. Monoclonal antibodies 3.6B and 5.6D bound to the human laminin B2 subunit in both its glycosylated and deglycosylated forms, and also bound to the deglycosylated, but not the glycosylated, EHS-

tumour laminin B2 subunit. Although 3.6B and 5.6D monoclonal antibodies labelled the deglycosylated murine laminin B2 subunit less intensely than did the 4.5D antibody and less intensely than they labelled the human laminin B2 subunit, this result demonstrates that removal of *N*-linked carbohydrates from murine EHS-tumour laminin at least partially unmasks the epitopes recognized by monoclonal antibodies 3.6B and 5.6D.

DISCUSSION

We have produced a series of monoclonal antibodies specific for the C-terminal region of the laminin B2 subunit that show no cross-reactivity with other laminin subunits or other proteins in Western blots of whole-tissue extracts. Some of these monoclonal antibodies cross-react with human and rat laminin. The region of the murine B2 sequence contained within XLB2.1 shares 88% amino acid similarity with the corresponding human sequence. It was surprising that more of our monoclonal antibodies (five) showed cross-reactivity between mouse and human laminin than between mouse and rat laminin (two), since we expected the two rodent molecules to be less divergent in evolution than the mouse and human molecules. However, the rat B2 subunit sequence has not yet been published.

We have shown that monoclonal antibodies 3.6B and 5.6D identify a region of the C-terminal portion of the B2 subunit that is antigenically different between mouse and man and, at least in part, this difference is due to different *N*-linked glycosylation patterns. Published sequences of the human and murine laminin B2 subunits (Sasaki & Yamada, 1987; Pikkarainen *et al.*, 1988) show an identical number and distribution of potential sites for *N*-linked glycosylation. Our results therefore suggest that either different potential glycosylation sites are occupied in the two species, or the oligosaccharides which occupy them are structurally different.

Surprisingly, there are no potential attachment sites for *N*-linked oligosaccharide within the B2 subunit sequence contained in XLB2.1, but there is a potential glycosylation site 16 amino acids *N*-terminal to this region. The *N*-linked sugars of murine EHS-tumour laminin are known to be large complex branched structures (Arumugham *et al.*, 1986) and the presence of an oligosaccharide at a distance may affect antibody binding by directly masking epitopes within the region encoded by XLB2.1, by imposing conformational constraints on the B2 subunit, which are lifted when the carbohydrate is removed, or by sterically hindering antibody binding.

As far as we are aware this is the first identification of a different usage of potential glycosylation sites in laminins from different origins. Preliminary results with rabbit antisera raised against XLB2.1 have suggested tissue, as well as species, differences in the glycosylation of laminin. This antiserum labelled bands of various sizes in different tissue extracts, the band labelled in brain and skin migrating faster than the band labelled in liver, kidney, skeletal muscle and spleen, which in turn migrated more rapidly than the band labelled in placenta, heart and lung (J. C. Brown, J. H. Spragg & P. W. Taylor, unpublished work). However, this is not the only possible explanation for the differences in mobilities seen on Western blots, and purified laminin from different tissues will be required to investigate this further.

The carbohydrate content of laminin is high, and Runyan *et al.* (1988) have shown that *N*-linked oligosaccharides are necessary for the spreading of B16-F10 melanoma cells. In addition, glycosylation affecting interactions with the region of the B2 subunit encoded in XLB2.1 may be of some significance, as Liesi *et al.* (1989) have recently identified a decapeptide from this region that promotes neurite outgrowth. Functional assays

using purified human laminin will be important in determining the effects of glycosylation patterns on biological activity and the extent to which data obtained using murine EHS-tumour laminin are applicable to laminin from other species.

REFERENCES

- Arumugham, R. G., Hsieh, T. C.-Y., Tanzer, M. L. & Laine, R. A. (1986) *Biochim. Biophys. Acta* **883**, 112–126
- Brown, J. C., Wheeler, G. N., Mathias, P. N., Spragg, J. H. & Taylor, P. W. (1989) *Biochem. Soc. Trans.* **17**, 185–186
- Brown, J. C., Spragg, J. H., Wheeler, G. N. & Taylor, P. W. (1990) *Biochem. J.* **270**, 463–468
- Chi, H. & Hui, C. (1988) *Nucleic Acids Res.* **16**, 7205–7206
- Chung, A. E., Jaffe, R., Bendr, B., Lewis, M. & Durkin, D. (1983) *Lab. Invest.* **49**, 576–581
- Engel, J., Odermatt, E. & Engel, A. (1981) *J. Mol. Biol.* **150**, 97–120
- Horikoshi, S., Koide, H. & Shirai, T. (1988) *Lab. Invest.* **58**, 532–538
- Liesi, P., Narvanen, A., Soos, J., Sariola, H. & Snounou, G. (1989) *FEBS Lett.* **244**, 141–148
- Montell, D. J. & Goodman, C. S. (1988) *Cell* (Cambridge, Mass.) **53**, 463–473
- Nissinen, M., Vuolteenaho, R., Boot-Handford, R., Kallunki, P. & Tryggvason, K. (1990) *J. Cell Biol. Suppl.* **111**, 400a (abstr.)
- Oi, V. T. & Herzenberg, L. A. (1980) in *Selected Methods in Cellular Immunology* (Mishell, B. B. & Shiigi, S. M., eds.), pp. 351–372, W. H. Freeman, San Francisco
- Olsen, D., Nagayoshi, T., Fazio, M., Peltonen, J., Jaakkola, S., Sanborn, D., Sasaki, T., Kuivaniemi, H., Chu, M.-L., Deutzmann, R., Timpl, R. & Uitto, J. (1989) *Lab. Invest.* **60**, 772–782
- Paulsson, M., Aumailley, M., Deutzman, R., Timpl, R., Beck, K. & Engel, J. (1987) *Eur. J. Biochem.* **166**, 11–19
- Pfaff, E., Mussgay, M., Boehm, H. O., Schulz, G. E. & Schaller, F. (1982) *EMBO J.* **1**, 869–874
- Pikkarainen, T., Eddy, R., Fukushima, Y., Byers, M., Shows, T., Pihlajaniemi, T., Saraste, M. & Tryggvason, K. (1987) *J. Biol. Chem.* **262**, 10454–10462
- Pikkarainen, T., Kallunki, T. & Tryggvason, K. (1988) *J. Biol. Chem.* **263**, 6751–6758
- Runyan, R., Versalovic, J. & Shur, B. (1988) *J. Cell. Biol.* **107**, 1863–1871
- Sasaki, M. & Yamada, Y. (1987) *J. Biol. Chem.* **262**, 17111–17117
- Timpl, R., Rohde, H., Gehron Robey, P., Rennard, S. I., Foidart, J. M. & Martin, G. R. (1979) *J. Biol. Chem.* **254**, 9933–9937

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