Structure and distribution of *N*-linked oligosaccharide chains on various domains of mouse tumour laminin

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Asparagine-linked oligosaccharides were liberated from laminin and some of its fragments by hydrazinolysis, and after purification characterized by exoglycosidase digestions. This demonstrated the presence of nine forms of complex oligosaccharide chains, which differed in antennary and oligolactosamine structure, and of small amounts of high-mannose-type oligosaccharides. Additional variations were found with regard to substitutions by terminal α -galactose and sialic acid residues. Each of the various laminin fragments showed a unique but less complex repertoire of carbohydrate structures. These fragments also differed in mass, carbohydrate content, localization within the laminin molecule and functional activities such as cell-binding (fragments 1 and 6) and heparin- and collagen-binding (fragments 3 and 4). Fragment 7 with a particularly high carbohydrate content (72%) also showed the highest complexity of tri- and tetra-antennary structures. Further differences between the fragments were detected with human antibodies against the Gal α 1-3Gal epitope, which was expressed in either a high-affinity or a low-affinity form. Such differences in carbohydrate structure of topologically distinct laminin domains may have implications for their functions and in the regulation of post-translational modification events.

INTRODUCTION

Laminin (M_r about 800 000) is known to be an ubiquitous basement-membrane protein and is most conveniently studied in the form produced by the mouse Engelbreth-Holm-Swarm (EHS) tumour (Timpl *et al.*, 1979). The protein possesses multiple biological activities, implicating a major functional role. These functions include anchorage of cells to basement membranes, presumably by means of two high-affinity cell-binding sites (Aumailley *et al.*, 1987; Goodman *et al.*, 1987; Graf *et al.*, 1987), and a variety of other molecular interactions within the extracellular matrix (reviewed by Timpl & Dziadek, 1986; Martin & Timpl, 1987). The diversity of functions is apparently reflected by the multidomain structure of laminin, which consists of several rod-like and globular domains assembled into a cruciform shape (Engel *et al.*, 1981; see Fig. 1). These domains are folded from segments of one or more constituent chains of laminin (B1, B2 and A; Cooper *et al.*, 1981), as also indicated from complete amino acid sequences available for both B chains (Sasaki *et al.*, 1987; Sasaki & Yamada, 1987). Several of these laminin domains have been isolated from proteolytic digests (Rao *et al.*, 1982; Ott *et al.*, 1982; Timpl *et al.*, 1983; Paulsson *et al.*, 1985), including two large cell-binding fragments 1 and 8 (M_r 200 000–300 000) and several smaller fragments comprising peripheral and internal domains. These fragments have assisted in mapping diverse biological functions within the protein (Martin & Timpl, 1987).

Another characteristic feature of laminin is its rather high carbohydrate content, of some 13–15% (Chung *et al.*, 1979; Engvall *et al.*, 1983; Aumailley *et al.*, 1983; Arumugham *et al.*, 1986). The high prevalence of glucosamine over galactosamine found, except in a single

Fig. 1. Schema of the multidomain structure of laminin and the localization of different proteinase-resistant fragments

The cell-binding fragments 1 (hatched area) and 6, heparin-binding fragment 3 and collagen-binding fragment 4 were characterized in previous studies (Ott *et al.*, 1982; Timpl *et al.*, 1983). The localization of fragment 7 is not determined.

study (Chung *et al.*, 1979), indicated the presence of many *N*-linked but not of *O*-linked oligosaccharides. This was also inferred from the tunicamycin-sensitivity of glycosylation (Cooper *et al.*, 1981; Howe, 1984) and the presence of many Asn-Xaa-Thr acceptor sites in both B chains (Sasaki *et al.*, 1987; Sasaki & Yamada, 1987). Arumugham *et al.* (1986) have isolated a set of *N*-linked glycopeptides from Pronase digests of EHS-tumour laminin and demonstrated a large variety of oligosaccharide structures. Nothing is known about the

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function of these carbohydrates, except that they may not be needed for heparin-binding or enhancing proteinase-stability of laminin (Howe, 1984). They possess, however, a distinct Gal α 1-3Gal epitope recognized by human antibodies (Towbin *et al.*, 1987; Galili *et al.*, 1987).

The various fragments of laminin differ considerably in carbohydrate content (8–72%; Aumailley *et al.*, 1983), suggesting that the number and structure of *N*-linked oligosaccharides of laminin domains may vary according to their localization and function. In the present study we subjected oligosaccharides from five different fragments of laminin, which accounted for 70% of total carbohydrate mass, to structural and immunological analyses and demonstrated that such variability among domains in fact exists. This suggests laminin glycosylation could have distinct functional consequences and that the glycosylation of different domains of laminin is topologically controlled.

MATERIALS AND METHODS

Proteins, enzymes and other reagents

Laminin (Timpl *et al.*, 1979) and some of its major fragments (Ott *et al.*, 1982; Timpl *et al.*, 1983) were purified from the EHS tumour. Fragments were obtained by cleavage with either pepsin (fragment 1) or elastase (fragments 3, 4, 6 and 7), and some were further purified on a TSK 3000 h.p.l.c. column. α -Mannosidase with β -mannosidase activity, β -*N*-acetylhexosaminidase and β -galactosidase were purified from jack-bean meal by the method of Li & Li (1972) and Arakawa *et al.* (1974). β -Galactosidase was purified from the culture medium of *Diplococcus pneumoniae* (Hughes & Jeanloz, 1964). Sialidase from *Arthrobacter ureafaciens* (Nakarai Chemicals, Kyoto, Japan), α -galactosidase from coffee bean (Boehringer, Mannheim, Germany) and α -L-fucosidase from *Charonia lampas* (Seikagaku Kogyo, Tokyo, Japan) were obtained commercially. NaB³H₄ (360 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and Bio-Gel P-4 (–400 mesh) was from Bio-Rad Laboratories (Richmond, VA, U.S.A.). Anhydrous hydrazine was prepared from hydrazine hydrate by distillation at 95 °C in the presence of toluene and CaO. All other chemicals used were of analytical grade.

Preparation of oligosaccharide standards

Biantennary Gal(β 1-4)GlcNAc(β 1-2)Man(α 1-6)-[(Gal(β 1-4)GlcNAc(β 1-2)Man(α 1-3)]Man(β 1-4)GlcNAc(β 1-4)GlcNAcitol lacking or possessing a terminal sialic acid were isolated from human complement subcomponent Clq (Mizuochi *et al.*, 1978). The mannose core structure was prepared from the first component by treatment with a mixture of β -galactosidase and hexosaminidase. Treatments of this product with β - and/or α -mannosidase produced the components Man(β 1-4)GlcNAc(β 1-4)GlcNAcitol and GlcNAc(β 1-4)GlcNAcitol respectively. Treatment of the sialylated starting component with β -galactosidase/hexosaminidase followed by sialidase enabled preparation of the mannose core containing a single lactosamine chain. Glucose oligomers were prepared from a dextran hydrolysate (Nishigaki *et al.*, 1978).

Liberation and fractionation of *N*-linked oligosaccharides

Protein-samples (20 mg/ml) were heated at 100 °C in anhydrous hydrazine for 12 h, and the reaction product was acetylated with acetic acid anhydride. Oligosaccharides were then labelled with NaB³H₄ (1–11 μ mol in 0.05 M-NaOH) at 30 °C for 4 h (Mizuochi *et al.*, 1978). Between these steps non-carbohydrate degradation products and reagents were removed by passage over Dowex 50W (H⁺ form) columns and by paper chromatography on Whatman 3MM paper with either ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.) or butan-1-ol/ethanol/water (4:1:1, by vol.) as solvent.

Radioactive oligosaccharides were separated on Whatman no. 1 paper by high-voltage electrophoresis at pH 5.4 in pyridine/acetic acid/water (3:1:3, by vol.) for 90 min at 73 V/cm. Radioactivity was monitored by radiochromatoscanner (model 7220, Packard Instruments), and pooled fractions were subjected to paper chromatography followed by elution with water and evaporation. Further purifications, if required, were done on Bio-Gel P-4 in conjunction with degradation experiments (see below).

Digestion with exoglycosidases

Various labelled oligosaccharide fractions (4×10^6 – 35×10^6 c.p.m.) obtained from 4–30 mg of protein were treated with several exoglycosidases for 16–24 h at 37 °C and then passed through a mixed column (0.5 cm $\times$ 1.5 cm) of Dowex 50W (H⁺ form) and Dowex 1-X2 (Cl[–] form) for removing salts and enzymes before Bio-Gel P4 chromatography. Digestions were carried out with sialidase (0.02 unit) in 20 μ l of 0.1 M-sodium acetate buffer, pH 4.5, with jack-bean β -galactosidase (2 units) in 50 μ l of 0.1 M-sodium citrate/phosphate buffer, pH 3.5, with hexosaminidase (1 unit) in 50 μ l of 0.1 M-sodium citrate/phosphate buffer, pH 5, with a mixture of *D. pneumoniae* β -galactosidase and hexosaminidase (each 1 unit) in 150 μ l of 0.1 M-sodium citrate/phosphate buffer, pH 3.5, with α -galactosidase (0.15 unit) in 30 μ l of 0.1 M-sodium citrate/phosphate buffer, pH 6, and with α -L-fucosidase (0.01 unit) at 50 °C in 50 μ l of 0.1 M-sodium citrate/phosphate buffer, pH 5. Digestions with mannosidase were performed in 0.1 M-sodium acetate buffer, pH 4.5, containing galactono-1,5-lactone (0.5 mg/0.1 ml) with either low amounts (0.4 unit/30 μ l; only α -mannosidase activity) or larger amounts (0.8 unit/70 μ l; both α - and β -mannosidase activity) of enzyme. One unit of glycosidase activity corresponds to the release of 1 μ mol of monosaccharide/min from the substrates.

Size determinations

The sizes of oligosaccharides and their degradation products were determined on a Bio-Gel P-4 column (2 cm $\times$ 100 cm), which was eluted at 50 °C with water with collection of 2.7 ml fractions at a flow rate of 18 ml/h. Radioactive samples were mixed with dextran hydrolysates or other calibrating carbohydrates which were detected by monitoring the effluent with a differential refractometer (Knauer type 98; Knauer G.m.b.H., Berlin, Germany). Positions of radioactive material were detected by liquid-scintillation counting (Beckman LS-9000 counter). Sizes were expressed as glucose equivalents, with residues of mannose, galactose and fucose

each contributing 1 equivalent and *N*-acetylhexosamines contributing 1.7–2.0 equivalents as shown with authentic oligosaccharide standards.

Amino acid sequencing

Edman degradation was performed in a gas-phase sequencer (model 470A, Applied Biosystems) or a spinning-cup liquid-phase sequencer (model 890C, Beckman), and liberated phenylthiohydantoin derivatives were identified by h.p.l.c. (Chu *et al.*, 1987). Trypsin cleavage of reduced and alkylated fragment 1 (4 h, 37 °C, enzyme/substrate ratio 1:100 by wt.) was followed by h.p.l.c. purification of peptides on a reversed-phase column.

Immunological assay

Terminal Gal α 1-3Gal structures were detected by radioimmunoassay with 125 I-labelled laminin and antisera specific for this epitope obtained from patients with American cutaneous leishmaniasis (Towbin *et al.*, 1987).

RESULTS

Complexity and fractionation of *N*-linked oligosaccharides from laminin and its fragments

Oligosaccharides were released by hydrazinolysis from laminin and from the purified fragments 1, 3, 4, 6 and 7 (Fig. 1) and labelled at their reducing ends to a specific radioactivity of 2×10^5 d.p.m./nmol by treatment with NaB 3 H $_4$. The amount of radioactivity recovered from

the various components allowed us to estimate the number of *N*-linked oligosaccharide chains to be about 38 for laminin, whereas as expected lower numbers were found for the fragments (Table 1). The number of chains in the fragments roughly paralleled the mass of carbohydrate present, indicating M_r values for the chains in the range 2000–5000.

The oligosaccharides could be separated by electrophoresis into a neutral fraction N and up to four acidic fractions (A1S, A1, A2S, A2) (Fig. 2), which occurred in approximately equimolar proportions in laminin (Table 1). The fragments frequently showed more simple patterns lacking one or several acidic fractions (fragments 1, 3 and 4; about 10 % carbohydrate). The carbohydrate-rich fragments 6 and 7 (50 % and 72 % respectively; Aumailley *et al.*, 1983), however, showed a high abundance of acidic oligosaccharides.

Further examination of the electrophoretically separated oligosaccharide fractions on Bio-Gel P-4 in some cases showed a single symmetric peak and simple degradation patterns (example given in Fig. 3b), indicating the presence of a single component. Other fractions revealed up to four peaks emerging from the column. Separated peaks showed after degradation the presence of either one or two components (Fig. 4), demonstrating a considerable carbohydrate heterogeneity for laminin and some of its fragments (Table 1). Together the data indicate a range in the size of oligosaccharides (expressed as glucose equivalents) between 12 and 25 residues, excluding sialic acid residues, which had to be removed before Bio-Gel P-4 chromatography (see below).

Table 1. Content and heterogeneity of *N*-linked oligosaccharides in laminin and its fragments

	Carbohydrate (approximate)		Relative amounts* after electrophoresis and size distribution (given in parentheses§)									
	$10^{-3} \times$ Total M_r *†	No. of chains†	N‡	A1S		A1		A2S		A2		
Laminin	100	38	1.0	$\begin{pmatrix} 19 \\ 14-16 \\ 12 \end{pmatrix}$	0.8	$\begin{pmatrix} 17-18 \\ 16 \\ 14-15 \end{pmatrix}$	0.8	$\begin{pmatrix} 19-23 \\ 16-17 \\ 14-15 \end{pmatrix}$	0.9	$\begin{pmatrix} 25-26 \\ 23-24 \\ 19 \\ 16-17 \end{pmatrix}$	0.7	$\begin{pmatrix} 19 \\ 16 \\ 14 \end{pmatrix}$
Fragment 1	22	8-10	1.0	$\begin{pmatrix} 15 \\ 14 \end{pmatrix}$			0.7	$\begin{pmatrix} 15 \\ 14 \end{pmatrix}$	0.4	$\begin{pmatrix} 19 \\ 17 \\ 16 \\ 14 \end{pmatrix}$	0.2	(N.D.)
Fragment 3	6	1-2	1.0	(15)			1.8	$\begin{pmatrix} 16-17 \\ 15 \\ 14 \end{pmatrix}$	0.6	(17)		
Fragment 4	6	2-3	1.0	$\begin{pmatrix} 16 \\ 15 \\ 14 \end{pmatrix}$			1.5	$\begin{pmatrix} 16 \\ 15 \\ 14 \end{pmatrix}$				
Fragment 6	15	3	1.0	(16)			5.2	(19)	9.1	(19)	9.1	(19)
Fragment 7	20	5-10	1.0	$\begin{pmatrix} 16-19 \\ 12 \end{pmatrix}$	1.5	$\begin{pmatrix} 25-26 \\ 22-23 \\ 19 \end{pmatrix}$ ¶	3.9	$\begin{pmatrix} 25-26 \\ 22-23 \\ 19 \end{pmatrix}$ ¶	3.2	(N.D.)	1.5	(N.D.)

* Calculated on the basis of previous data (Aumailley *et al.*, 1983; Timpl *et al.*, 1986) and assuming M_r 800 000 for laminin.

† Determined from specific radioactivity recovered after hydrazinolysis and reduction with NaB 3 H $_4$.

‡ Amount of material in neutral fraction N set at 1 (see Fig. 2).

§ Determined by molecular-sieve chromatography (see Figs. 3 and 4) of desialylated components and expressed as glucose equivalents (rounded off to the nearest integer). Abbreviation: N.D., not determined.

|| and ¶ Data obtained from combined fractions.

Fig. 2. Paper electrophoresis of *N*-linked oligosaccharides liberated from laminin by hydrazinolysis

Components were labelled at their reducing ends with NaB^3H_4 and separated into a neutral (N) and several acidic (A) fractions. BPB indicates the migration position of Bromophenol Blue.

Structure of oligosaccharides

Various oligosaccharides purified from laminin fragments, as described above, were subjected to several digestions with exoglycosidases used in various combinations and sequences and the products were analysed by

chromatography on a high-resolution Bio-Gel P-4 column. Similar approaches have permitted analyses of *N*-linked carbohydrate structures present on various serum (Mizuochi *et al.*, 1978, 1982; Yoshima *et al.*, 1981) and extracellular-matrix proteins (Shinkai *et al.*, 1983; Shinkai & Lapiere, 1983). This procedure led to the identification of nine basic complex oligosaccharides (numbered I to IX) on laminin fragments that differ in the number and length of lactosamine side chains and a minor fraction of mannose-rich oligosaccharides (Table 2). Parallel analysis of oligosaccharides obtained from laminin failed to identify any other basic structures. It was also apparent that the various fragments of laminin possessed these carbohydrate structures in various combinations (Table 2).

Examples for this analytical procedure are illustrated in Fig. 3 for two biantennary structures. One component was treated successively with β -galactosidase, hexosaminidase and α -/ β -mannosidase and lost from its original 14 residues two, four (equivalent to two hexosamine residues) and three residues respectively, this resulting in a basic five-residue structure (Fig. 3a) corresponding to $\text{GlcNAc}(\beta 1-4)[\text{Fuc}(\alpha 1-2)]\text{GlcNAc}(\text{ol})$ (see below). Another 15-residue component lost only one and two residues respectively after treatments with β -

Fig. 3. Change in elution position on Bio-Gel P-4 after sequential exoglycosidase treatment of biantennary complex oligosaccharides obtained from A1 fraction of laminin fragment 1 (a) or N fraction of laminin fragment 3 (b)

Both starting materials shown in (a) and (b) were initially treated with β -galactosidase (profile I) followed by hexosaminidase (profile II). Material obtained in profile II in (a) was subsequently treated with α - and β -mannosidase (profile III in a). Material of profile II in (b) was successively treated with α -galactosidase (profile III in b), β -galactosidase (profile IV in b) and hexosaminidase (profile V in b), including column purification after each step. Profile IV in (a) shows a separate experiment where the component in (a) was first treated with β -galactosidase followed, after heat inactivation, with sialidase. The Bio-Gel P-4 column (2 cm $\times$ 100 cm) was calibrated by a series of glucose oligomers denoted at the top by numbers and by some authentic branched oligosaccharides (a-d; see the Materials and methods section): a, biantennary sugar with two lactosamine groups and after removal of one (b) or both (c) of these units; d corresponds to $\text{Man}(\beta 1-4)\text{GlcNAc}(\beta 1-4)\text{GlcNAc}(\text{ol})$; V_0 denotes the void volume. Radioactivity is given in arbitrary units. The components identified by these experiments correspond to structure I in Table 2 possessing either a single sialic acid (a) or α -galactose residue (b) at one non-reducing end.

Fig. 4. Heterogeneity of electrophoretically separated oligosaccharides revealed by Bio-Gel P-4 chromatography of A1 fraction from laminin fragment 6 (a) and A2S fraction from laminin fragment 1 (b), both in the top profiles

The bottom profiles show the respective materials when the first peaks separated in the top profile were digested with β -galactosidase. Calibration of the column is the same as shown in Fig. 3.

Table 2. Proposed oligosaccharide structures obtained from laminin fragments

R = Man-GlcNAc-GlcNAc-ol; R' = Man-GlcNAc-(Fuc-)GlcNAc-ol.

	Structure	Presence on laminin fragment					Presence of terminal		Presence in electrophoretic fraction (laminin fragment)
		1	3	4	6	7	α -Gal	Sia	
I	Gal-GlcNAc-Man						+	+	A1 (1, 3, 4)
	Gal-GlcNAc-Man-R'	+	+	+	-	-	+	-	N (1, 3, 4)
							-	+	A1 (1, 3, 4), A2S (1)
II	Gal-GlcNAc-Gal-GlcNAc-Man						-	+	A2S (1)
	Gal-GlcNAc-Man-R'	+	-	-	-	+	-	-	N (7)
							-	-	
III	(Gal-GlcNAc) ₂ -Gal-GlcNAc-Man-R'	-	-	-	-	+	-	-	N (7)
IV	Gal-GlcNAc-Man						+	+	A1 (3), A2S (1, 3)
	Gal-GlcNAc-Man-R'	+	+	+	+	-	-	+	A1 (3, 4), A2S (1)
	Gal-GlcNAc						-	-	N (4, 6)
V	(Gal-GlcNAc)-Gal-GlcNAc-Man						-	+	A1S-A1 (7), A2S (1)
	-Gal-GlcNAc-Man-R'	+	-	-	-	+	-	-	N (7)
	-Gal-GlcNAc						-	-	
VI	(Gal-GlcNAc) ₂ -Gal-GlcNAc-Man-R'	-	-	-	-	+	-	+	A1S-A1 (7)
	-Gal-GlcNAc-Man						-	+	
							-	+	
VII	Gal-GlcNAc						-	+	A1-A2 (6)
	Gal-GlcNAc-Man-R'	+	-	-	+	+	-	+	A1S-A1 (7), A2S (1)
	Gal-GlcNAc-Man						-	+	
VIII	(Gal-GlcNAc)-Gal-GlcNAc						+	+	A1S-A1 (7)
	-Gal-GlcNAc-Man-R'	-	-	-	-	+	-	+	A1S-A1 (7)
	-Gal-GlcNAc-Man						-	+	
IX	(Gal-GlcNAc) ₂ -Gal-GlcNAc-Man		-	-	-	+	+	+	A1S-A1 (7)
	-Gal-GlcNAc-Man-R'						-	+	A1S-A1 (7)
	-Gal-GlcNAc						-	+	
X	Man-Man						-	-	
	Man-Man-Man-R	-	-	-	-	+	-	-	N (7)
	Man-Man						-	-	

galactosidase and hexosaminidase (Fig. 3b). Sensitivity to both exoglycosidases could be generated again after treatment with α -galactosidase, indicating that one lactosamine side chain was blocked by α -glycosidically

linked galactose. Similar repeated digestion experiments were used to determine the number of free and blocked lactosamine side chains in tri- and tetra-antennary oligosaccharides (results not shown).

A complication of this type of analysis arose from the fact that carbohydrates in laminin (Arumugham *et al.*, 1986) may contain oligolactosamine side chains. Such components were identified as structures II, III, V, VI, VIII and IX (Table 2) and were particularly, but not exclusively, found among the oligosaccharides of fragment 7. Their presence was indicated by their unusual size on Bio-Gel P-4, exceeding those of simple bi- to tetra-antennary oligosaccharides (structures I, IV and VIII), but could, however, by sequential treatment with β -galactosidase and hexosaminidase from jack bean (including if required pretreatments with sialidase and α -galactosidase) all be converted into two basic structures corresponding to 11 or 14 glucose equivalents on the Bio-Gel P-4 column. Further sequential degradations with β -galactosidase and hexosaminidase revealed that in each component one antenna was extended by one (structures II, V and VII) or two (structures III, VI and IX) more lactosamine units (results not shown). The degradation data did not permit distinction of to which particular mannose residue this oligolactosamine was conjugated. Treatment of fraction N obtained from laminin with a mixture of β -galactosidase and β -N-acetylhexosaminidase from *D. pneumoniae* converted all material into 8 glucose equivalents. This indicates that galactose residues in the branching chains are connected to N-acetylglucosamine in β 1-4-linkage (Paulson *et al.*, 1978).

Laminin also contains a substantial number of terminal α -galactose residues, as shown by lectin binding, by release with α -galactosidase (Shibata *et al.*, 1982; Rao *et al.*, 1983; Arumugham *et al.*, 1986) and by immunological assays (Towbin *et al.*, 1987). Their presence on oligosaccharides was identified after treatment with α -galactosidase, resulting in a shift on the Bio-Gel P-4 column corresponding to 1 glucose equivalent (Fig. 3b), which usually rendered the product more susceptible for β -galactosidase treatment, indicating α -glycosidic linkage to terminal lactosamine structures. As found for oligosaccharide fraction N obtained from fragment 3 (Fig. 3), all of the biantennary oligosaccharide (structure I, 15 glucose equivalents) was substituted by a single α -galactose residue. The same fraction N obtained from fragment 4 contained two biantennary components (equivalent to 15 and 14 glucose residues respectively), demonstrating that about half of this material did not possess a terminal α -galactose residue. These α -galactose residues were also detected in more complex oligosaccharides (Table 2) and were usually represented by a mixture of substituted and non-substituted variants.

Oligosaccharides of laminin and the fragments characterized here contain significant amounts of sialic acid, which was particularly prominent in fragments 6 and 7 (Aumailley *et al.*, 1983; Engvall *et al.*, 1983). This agrees with the abundance of acidic oligosaccharides (Table 1) and the distinct heterogeneity observed after electrophoresis (Fig. 2). All the acidic components but not the neutral ones of fraction N were eluted anomalously in the void volume of the Bio-Gel P-4 column, as a consequence of their negative charge. Prior treatment with sialidase was therefore required to analyse these components and to reveal their heterogeneity of core structures (Figs. 3a and 4). Sialylation may occur only at a single lactosamine unit, as shown for the biantennary structure I obtained from fragment 1. This was revealed by the release of either one or two residues by β -galactosidase from the oligosaccharide if digestion was

done either before or after desialylation (Fig. 3a, compare profiles I and IV). Substitutions with sialic acid of a particular oligosaccharide may also not be complete, as found for fractions N and A1 obtained from fragment 4, which are present in similar amounts (Table 1) and contain the same two biantennary carbohydrates (see above) either in non-sialylated or sialylated form. These and other data indicate a considerable diversity in sialylation patterns (Table 2).

Extensive degradations with sialidase, galactosidases, hexosaminidase and mannosidases led for all complex oligosaccharides to a core structure corresponding on Bio-Gel P-4 to 5 glucose equivalents (Fig. 3a, profile III). Further treatment with α -L-fucosidase released one more glucose equivalent, indicating that fucose was connected to the N-acetylglucosamine residue involved in the binding to asparagine. Small amounts of fucose were previously found by compositional analyses (Chung *et al.*, 1979; Aumailley *et al.*, 1983; Engvall *et al.*, 1983) and could account for this general modification (Table 2). These previous compositional analyses also demonstrated some glucose, which apparently is not present in the complex oligosaccharides, on the basis of the degradation experiments. It very probably arose as a contaminant from column material during the purification of laminin and its fragments.

Mannose-rich oligosaccharides could only be detected in fragment 7, where they accounted for about 20% of the carbohydrate present in fraction N. The material appeared as a symmetrical peak on Bio-Gel P-4, corresponding to 12 glucose equivalents before and to about 5 equivalents after extensive digestion with small amounts of mannosidase. One more residue could be removed by exposure to large amounts of mannosidase but not to α -L-fucosidase. This led to the tentative structure X listed in Table 2.

Distribution of galactosyl-(α 1-3)-galactose epitopes

These terminal epitopes can be detected by human antibodies present in normal and patient sera and analysed in a quantitative manner by laminin radioimmuno-inhibition assay. The antibodies are also rather specific and fail to cross-react significantly with related structures such as Gal α 1-4Gal, Gal α 1-6Gal and Gal α 1-3(Fuc α 1-2)Gal (Towbin *et al.*, 1987). This assay was used to compare laminin with its fragments (Fig. 5). The data revealed equal activity for laminin and fragments 1 and 4 and a 2-fold higher activity for fragment 3. These inhibitors produced inhibition curves with identical slopes, indicating that the epitopes have a comparable affinity for antibodies. Much less steep inhibition profiles were, however, observed for the carbohydrate-rich fragments 6 and 7, which we interpret as showing a smaller number of epitopes and/or a distinctly lower affinity for antibodies.

Identification of fragments by amino acid sequence analysis

The topological position of most of the fragments studied here within the cross-shaped laminin structure (Engel *et al.*, 1981; Fig. 1) is known, but not so far their origin from different segments within the laminin B1, B2 and A chains. This was approached by limited amino acid sequence analysis of the fragments (Fig. 6). A unique single N-terminal sequence was found for both fragments 3 and 4, demonstrating that they consist of a

Fig. 5. Detection of galactosyl-(α 1-3)-galactose epitopes on laminin fragments by radioimmunoassay

The test system consisted of 125 I-labelled laminin and serum from a patient with American cutaneous leishmaniasis containing high-titre antibodies against this epitope (Towbin *et al.*, 1987). Inhibitors used in the reaction were laminin (○) and its fragments 1 (●), 3 (△), 4 (▲), 6 (□) and 7 (■). Inhibitor concentrations are expressed as carbohydrate equivalents (Aumailley *et al.*, 1983).

Fragment 1:	A E G L S L Q P Y E E Y F D V	(A)
	S C Y Q D P V T L Q L A C V C	(B1)
	P S A Y N F D N S P V L	(B2)
Fragment 3:	L H R E H G E L P P E P P T L	(A)
Fragment 4:	Y G C A E G S C Y P A T G D L	(B1)

Fig. 6. Identification of laminin fragments 1, 3 and 4 by characteristic amino acid sequences

The sequences were obtained from the N-termini of fragments 3 and 4 and from several tryptic peptides of fragment 1. Their origin from either laminin B1, B2 or A chain is indicated in parentheses.

single polypeptide. The fragment 4 sequence corresponds to positions 7–21 at the N-terminus of laminin B1 chain (Sasaki *et al.*, 1987). The fragment 3 sequence matched a nucleotide sequence in a cDNA clone corresponding to the 3' end of A-chain mRNA (R. Deutzmann, unpublished work). Since fragment 1 possesses several N-terminal amino acid residues (Rohde *et al.*, 1980), it was cleaved by trypsin and h.p.l.c.-purified peptides were subjected to Edman degradation. Several of the peptides matched sequences known for the central portion of B1 and B2 chains (Sasaki *et al.*, 1987; Sasaki & Yamada, 1987), for which examples are shown. Several other peptides did not match B1 or B2 structures but were also found among the tryptic peptides produced from laminin A chain (R. Deutzmann, unpublished work). This demonstrates that fragment 1 originates from all three laminin chains. Heterogeneous sequence data were obtained for fragments 6 and 7, indicating their release from laminin by multiple proteolytic cleavages.

DISCUSSION

Laminin from the EHS mouse tumour possesses some 40 N-linked oligosaccharides as determined in our and a previous study (Arumugham *et al.*, 1986). This number is in good agreement with 12 and 14 potential Asn-Xaa-Thr acceptor sites present in the B1 chain and B2 chain respectively (Sasaki *et al.*, 1987; Sasaki & Yamada, 1987) and a presumed equal or higher content in the larger A chain, indicating that most of these acceptor sites are actually used for glycosylation. The basic structures involved comprise nine different complex types with regard to antennary and oligolactosamine units, which are further diversified by terminal α -galactose and sialic acid residues. Additionally, small amounts of mannose-rich oligosaccharides could be detected. The diversity of structures could be due to the fact that the analysed laminin was a tumour-cell product (Muramatsu *et al.*, 1982, 1983), but comparable amounts of laminin from authentic basement membranes are not yet available to study this question. However, nidogen, another protein from the EHS tumour, with 5% carbohydrate (Paulsson *et al.*, 1986), contains a much less diverse repertoire of oligosaccharide structures (S. Fujiwara, unpublished work), indicating that overglycosylation is not a general phenomenon in the EHS tumour.

The carbohydrate structures described here agree with those published previously (Arumugham *et al.*, 1986), which were determined by somewhat different procedures, including methylation analysis. Our data extend the previous ones by a topological analysis with domain-specific fragments, a more precise analysis of lactosamine oligomers, the identification of fucose in the core structures and the localization of some of the terminal α -galactose and sialic acid residues.

The various N-linked oligosaccharides do not appear to be randomly distributed within the molecule, but rather show a prevalence of biantennary structures on peripheral domains (fragments 3 and 4) and of more complex structures on the carbohydrate-rich fragments 6 and 7. As shown in the present work, fragment 4 originates from the N-terminus of the B1 chain and constitutes a globular and rod-like domain at the end of one short arm of laminin (Ott *et al.*, 1982; Timpl *et al.*, 1983). This segment contains three potential Asn-Xaa-Thr acceptor sites (Sasaki *et al.*, 1987). Fragment 3 constitutes a portion of the large globular domain at the end of the long arm (Ott *et al.*, 1982), originating from some 400 amino acid residues including three acceptor sites at the C-terminus of the A chain (R. Deutzmann, unpublished work). The small number of acceptor sites agrees with a comparable number of oligosaccharide chains found on the two fragments (Tables 1 and 2). Both domains have been implicated in laminin binding to collagen type IV (Rao *et al.*, 1982; Charonis *et al.*, 1985, 1986) and to heparin and heparan sulphate proteoglycan (fragment 3 only; see Ott *et al.*, 1982; Fujiwara *et al.*, 1984). Whether the carbohydrates present on these domains contribute to binding activity remains to be studied.

Fragment 1 of laminin comprises a central portion of the cross-shaped molecules (Engel *et al.*, 1981; Ott *et al.*, 1982) and contains peptide sequences of all three constituent chains (Fig. 6). These sequences are folded into repeating cysteine-rich motifs, with some similarity to epidermal growth factor (Sasaki *et al.*, 1987; Sasaki &

Yamada, 1987). They may contain some ten Asn-Xaa-Thr sites, in agreement with a complex repertoire of carbohydrate structures. Fragment 1 is known to contain major high-affinity cell-binding sites (Rao *et al.*, 1982; Timpl *et al.*, 1983; Graf *et al.*, 1987; Aumailley *et al.*, 1987). Since cell-matrix interactions represent rather complex processes (Timpl & Dziadek, 1986), peptide sequences (Graf *et al.*, 1987) as well as carbohydrate structures (Dennis *et al.*, 1984) may be involved in cellular recognition of laminin. Another, minor, cell-binding fragment 6 of laminin (Timpl *et al.*, 1983) contains some but not all of the oligosaccharides also present in fragment 1. The most diverse oligosaccharide structure, including seven different basic types of chains, was detected on fragment 7, which contains about 70% carbohydrate (Aumailley *et al.*, 1983). Neither the functions nor the origin of this fragment have so far been elucidated.

Mouse laminin also contains several Gal α 1-3Gal epitopes (Towbin *et al.*, 1987), which can be recognized by human antibodies of rather narrow specificity present in normal and patient sera (Galili *et al.*, 1987; Towbin *et al.*, 1987). Studies with laminin fragments demonstrated that the epitopes exist in apparently high-affinity and low-affinity form. Compared with laminin high affinity was detected on fragments 1, 3 and 4, which agrees with the demonstration of distinct amounts of terminal α -galactose by chemical analysis. Because of the high specificity of the immunological reactions, it also indicates an α 1-3-linkage for these residues. Fragment 7, however, with a similar content of α -galactose (about 10% of chains), shows low affinity, as indicated by a shallow inhibition profile (Fig. 5). Fragment 6 contains less α -galactose (< 5% substitution), resulting in lower inhibitory potency of the low-affinity type. This could indicate that fragments 6 and 7 possess terminal galactose in a α -glycosidic linkage different from that in the more active fragments. Alternatively, the high density of oligosaccharides on fragments 6 and 7 (Aumailley *et al.*, 1983) may for steric reasons interfere with antibody binding, thus mimicking low affinity.

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