Structure of the Major Concanavalin A Reactive Oligosaccharides of the Extracellular Matrix Component Laminin†

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ABSTRACT: Laminin, a high molecular weight (1 000 000) glycoprotein component of basement membranes, was isolated from the EHS murine tumor as a noncovalent complex with entactin by lectin affinity chromatography using the α-D-galactosyl binding lectin *Griffonia simplicifolia* I (GS I). Entactin was removed from this complex by passage over Sephacryl S-1000 in the presence of SDS. Compositional analysis showed that the affinity-purified laminin contained 25–30% carbohydrate by weight. Methylation analysis revealed that the oligosaccharides of laminin contained bi- and triantennary chains, the blood group I structure, and repeating sequences of 3Galβ1,4GlcNAcβ1 units. Free oligosaccharides were derived from the asparagine-linked glycans of affinity-purified laminin by hydrazinolysis, re-N-acetylation, and reduction with NaB^3H_4. When fractionated by affinity chromatography on concanavalin A (Con A)–Sepharose, 80% of the oligosaccharides passed through the column unretarded and a single peak corresponding to 20% of the oligosaccharides was adsorbed and specifically eluted with a linear gradient of 0–30 mM methyl α-D-glucopyranoside. Further fractionation of the Con A reactive oligosaccharides on GS I–Sepharose demonstrated that 70% of these oligosaccharides possess at least one terminal nonreducing α-D-galactopyranosyl unit. The Con A reactive oligosaccharides were subjected to sequential digestion with endo- and exoglycosidases, and the reaction products were analyzed by gel filtration chromatography on a column of Bio-Gel P4. We thereby obtained evidence for a variety of structures not previously reported to exist on murine laminin including hybrid biantennary complex and biantennary complex structures containing poly(lactosaminyl) repeating units. The poly(lactosaminyl) units occur either on one or on both branches of the biantennary chains, as well as in more highly branched blood group I poly(lactosamine) structures. All sialic acid is present as *N*-acetylneuraminic acid linked α2,3 to galactose.

Laminin is a high molecular weight glycoprotein that occurs in the basement membranes of a variety of tissues, including vascular basement membranes (Becker et al., 1986; Gordon & Essner, 1986), skin (Ekblom et al., 1982; Lane et al., 1985), kidney glomerulus (Abrahamson, 1985; Jaffe et al., 1984; Abrahamson & Caulfield, 1985; Ekblom et al., 1982), muscle (Sanes et al., 1986), liver (Sell & Ruoslahti, 1982), and nerve tissue (Palm & Furcht, 1983; Rogers et al., 1983, 1986; Bignami et al., 1984), and has been isolated in intact form from the EHS murine tumor (Timpl et al., 1979) and rat yolk sack tumor (Chung et al., 1979). Laminin has been shown to bind specifically to a number of extracellular matrix components, including type IV collagen (Terranova et al., 1980), heparan sulfate proteoglycan (Del Rosso et al., 1981), and entactin/nidogen (Timpl et al., 1983) as well as two classes of cell surface receptors (Malinoff & Wicha, 1983; Huard et al., 1986; Lesot et al., 1983; Terranova et al., 1983). Models have been proposed to explain the function of laminin in the extracellular matrix and its potential role in tumor metastasis (Liotta et al., 1983). These models are focused on the ability of laminin to promote cell attachment and cell spreading in vitro and its role as an attachment factor for normal and transformed cells in vivo. Studies on the characterization of laminin-mediated adherence of cells (Barsky et al., 1984; Fligiel et al., 1985) and reports of the ability of laminin to confer metastatic potential to poorly metastatic cells (Terranova et al., 1984; Malinoff et al., 1984; Varani et al., 1985) have supported these models.

A clear understanding of the biological function of laminin requires a knowledge of its structure. In this paper we have investigated the carbohydrate structure of laminin from the EHS tumor. These studies are focused on a subset of the N-linked oligosaccharides of laminin that bind specifically to Con A[1]–Sepharose. The abundance of carbohydrate and the structures reported here stand in contrast to a previous paper describing the oligosaccharides present on the laminin molecule from the EHS tumor (Arumugham et al., 1986).

Materials and Methods

Materials. D-Galactose, methyl α-D-galactopyranoside, and 2-deoxy-2-acetamido-D-glucopyranose were obtained from Pfanstiehl Laboratories (Waukegan, IL). Bio-Gel P series resins were obtained from Bio-Rad (Richmond, CA). Sepharose, Sephadex, QAE-Sephadex, Sephacryl, fluorescein isothiocyanate, and PMSF were obtained from Sigma Chemical Co. Freund's complete adjuvant, Freund's incomplete adjuvant, and complete H37Ra adjuvant were obtained from Difco Laboratories (Detroit, MI). *N*-(Acetylmuramyl)-L-alanyl-D-isoglutamine was purchased from Calbiochem (La Jolla, CA). Acetonitrile was redistilled over phosphorus pentoxide and stored over 3-Å molecular sieves under nitrogen.

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[1] Abbreviations: PBS, phosphate-buffered saline, pH 7.2; endo H, endo-β-*N*-acetylglucosaminidase H; endo C, endo-β-galactosidase C; Con A, concanavalin A; GS I, *Griffonia simplicifolia* I isolectins; $NaPO_4$, sodium phosphate; LDS, lithium dodecyl sulfate.

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Anhydrous hydrazine (Aldrich Chemical Co.) was redistilled over calcium oxide under nitrogen immediately prior to use.

Coffee bean α-galactosidase, β-galactose dehydrogenase, and β-nicotinamide adenine dinucleotide were purchased from Boehringer Mannheim Biochemicals. Endo-β-*N*-acetylglucosaminidase H was the gift of Dr. F. Maley, and peptide *N*-glycanase F was the gift of Dr. A. Tarentino, both of the New York State Department of Health, Albany, NY. *Escherichia freundii* endo-β-galactosidase was generously provided by Dr. Y. T. Li of Tulane University and by Dr. M. Fukuda of the La Jolla Cancer Research Foundation. *Clostridium perfringens* neuraminidase and bovine testicular β-galactosidase were the gifts of Dr. G. W. Jourdian of the University of Michigan Medical School. New Castle disease virus neuraminidase was the gift of Dr. J. U. Baenziger of Washington University Medical School, St. Louis, MO. Endo-β-galactosidase C was the gift of Dr. N. Fushuka. Other exoglycosidases were obtained from Boehringer Mannheim or Sigma.

The oligosaccharide standards $(Man)_{5-9}GlcNAc$ were the gift of Dr. B. P. Peters, Department of Pharmacology, University of Michigan Medical School. The $(Man)_1GlcNAc_2$–O^3H standard was the gift of Dr. A. Kobata of the University of Tokyo, Japan. Anti-entactin antiserum was kindly provided by Dr. A. Chung of the University of Pittsburgh. The lectin from *Maackia amurensis* was generously provided by Dr. R. D. Cummings, University of Georgia, Athens, GA. Con A–Sepharose and reagent antibodies conjugated to alkaline phosphatase were the generous gifts of Dr. A. Chu of E. Y. Laboratories (San Mateo, CA).

Assay Procedures. Protein concentrations were determined by the method of Lowry et al. (1951). Neutral sugars were determined by the phenol–sulfuric acid colorimetric assay of Dubois et al. (1956). The sialic acid content of glycoproteins, glycopeptides, and oligosaccharides was determined by the assay of Warren (1959).

Release of galactosyl residues following exoglycosidase digestion was determined by the galactose dehydrogenase assay (Finch et al., 1969). Amino acid analysis was performed by the method of Koop et al. (1981). Analysis of neutral and amino sugars in glycoprotein and oligosaccharide samples was performed as previously described (Perini & Peters, 1982). Specific identification of sialic acid was determined by the method of Schauer (1978).

Laminin Purification and Criteria of Purity. Griffonia simplicifolia I isolectins were purified as previously described (Delmotte & Goldstein, 1980). The GS I–Sepharose affinity matrix was synthesized as follows: Sepharose 4B was activated with cyanogen bromide (March et al., 1974) and mixed with GS I isolectins and protective haptenic sugar (100 mM methyl α-D-galactoside) overnight at 4 °C. Greater than 98% of the lectin was coupled to the activated Sepharose by this procedure, with a lectin density of 3–4 mg of lectin/mL of Sepharose for the affinity chromatographic purification of laminin. Affinity resins used in the fractionation of oligosaccharides were synthesized with a lectin density of 6–8 mg of lectin/mL of Sepharose.

The EHS murine sarcoma was grown as a solid tumor in C57B16 female mice (Orkin et al., 1977). Laminin was isolated from the EHS tumor by use of the GS I–Sepharose affinity column as previously described by Shibata and coworkers (Shibata et al., 1982). Typically, 5–6 mg of laminin was obtained per gram wet weight of tumor.

Further purification of laminin from its complex with entactin was performed as follows: 30 mg of affinity-purified protein solution in 20 mL of a buffer containing 0.1% LDS, 0.6% LiCl, 50 mM Tris, pH 7.2, and 0.04% sodium azide was heated at 95 °C for 10 min to denature the proteins. This material was applied to a Sephacryl S-1000 superfine column (2.7 × 100 cm) equilibrated in 50 mM Tris, pH 7.2, containing 0.1% LDS, and 3.0-mL fractions were collected. Protein was located by absorbance at 280 nm and analyzed by SDS–PAGE by the procedure of Laemmli (1970). Protein bands were detected by the silver stain technique of Morrissey (1981).

Immunochemical Procedures. Antibodies to affinity-purified laminin were raised in New Zealand white rabbits as previously described (Lapresle & Goldstein, 1969); 50 μg of antigen in a total volume of 100 μL was injected into each of three rabbits. Agar gel double diffusion was carried out as previously described (Oudin, 1980). Dot immunoblot assays were performed as described previously (Hawkes et al., 1982; Beyer, 1984).

Methylation Analysis. Methylation of laminin oligosaccharides was performed according to the method described by Ciucanu and Kerek (1984). Hydrolysis, reduction, and acetylation were carried out as described by by Stellner et al. (1973).

Purified oligosaccharide, 0.5–1 mg, was dried over P_2O_5 under reduced pressure for 48 h. Dimethyl sulfoxide (Sigma) was redistilled over NaOH pellets under reduced pressure; NaOH was powdered and dried under vacuum over P_2O_5 prior to use. Reagent grade methyl iodide (Aldrich) was used without further purification.

Methylation was accomplished by the sequential addition of 50 μL of DMSO, dried NaOH (4 mg), and 25 μL of methyl iodide to the purified oligosaccharide. The reaction was carried out at room temperature for 20 min with stirring. This procedure was repeated three times. The reaction was stopped by the addition of water, and methylated oligosaccharides were extracted with chloroform and dried under nitrogen. The methylated oligosaccharides were hydrolyzed at 100 °C for 6 h in 1 mL of 4 N trifluoroacetic acid. Aqueous methanol was added, and the sample was evaporated to dryness until neutral pH was reached. The methylated monosaccharides were dissolved in 5% NH_4OH and reduced with 50 μL of 0.22 M $NaBH_4$ at 25 °C overnight. The reduced carbohydrates were acetylated at 100 °C for 2 h by the addition of 1.5 mL of acetic anhydride. Acetylation was repeated twice. The methylated, peracetylated carbohydrates were analyzed on a fused silica glass capillary column (1 μm × 25 m, 007 series, methyl silicone, Quadrex Corp.) by using a Perkin-Elmer Model 8500 gas chromatograph with a temperature program (5 min at 130 °C, increasing at 5 °C/min, 10 min at 165 °C, 5 °C/min to 185 °C, hold for 10 min). The identity of standards and major peaks was confirmed by gas chromatography–mass spectrometry and was performed by Dr. M. Slodki of the USDA, Peoria, IL, Dr. C. Sweeley of the Michigan State University, East Lansing, MI, and Dr. Reddy of the State University of New York, Buffalo, NY.

Hydrazinolysis. Laminin (50 mg) was precipitated from PBS by adjusting the solution to a final concentration of 0.1 M HCl, 80% acetone. The precipitate was collected and dried in a vacuum oven over phosphorus pentoxide. Anhydrous hydrazine was refluxed over CaO overnight under a nitrogen atmosphere and then distilled. Freshly distilled hydrazine (0.5 mL) was added to the laminin sample; the reaction tube was sealed under N_2 and heated at 105 °C for 8 h. Hydrazine was partially removed by evaporation at reduced pressure. Oligosaccharides were re-N-acetylated by adding 1 mL of saturated sodium bicarbonate solution and a 20-fold molar excess

of acetic anhydride, relative to amino sugars. The reaction mixture was lyophilized and desalted on a Bio-Gel P2 column (1.5 × 95 cm) eluted with distilled water. The phenol–sulfuric acid positive, hexose-containing peak eluting in the void volume was collected. The presence of phenol–sulfuric acid positive peaks, partially included when desalted, was indicative of incomplete re-N-acetylation. Complete re-N-acetylation required six additions of acetic anhydride. The acetylated oligosaccharides were lyophilized, reconstituted in 0.1 M NaOH, reduced with 1 mCi of NaB^3H_4 (200 mCi/mmol) in 50 μL dimethylformamide overnight at room temperature, and desalted. Peaks were located by scintillation counting. To determine if the oligosaccharides had been degraded during hydrazinolysis, all phenol–sulfuric acid positive fractions were spotted on a silica gel 60-F254 TLC plate (Merck) and developed twice in ethyl acetate/acetic acid/water (3/2/2 v/v/v). The dried plate was scraped in 1-cm segments for the length of the plate, and radioactivity of these segments was determined by counting in Safety Solve (RPI) scintillation fluid. Radioactivity that migrated from the origin was indicative of degradation of the oligosaccharides.

Fractionation of Laminin Oligosaccharides by Lectin Affinity Chromatography. The mixture of [^{3}H]laminin oligosaccharides was applied to a Con A–Sepharose (0.9 × 50 cm) affinity column, the unbound material being eluted with 2 column volumes of PBS buffer. The bound material was displaced by using a linear gradient of 0–50 mM Me α-D-Glc*p*, followed by 2 column volumes of 100 mM Me α-D-Man*p* to ensure complete removal of bound oligosaccharides. The concentration of eluting sugar throughout the gradient was determined by the phenol–sulfuric acid assay. Oligosaccharide peaks were located by scintillation counting.

Both the Con A bound and Con A unbound fractions were further fractionated on an affinity column of GS I–Sepharose 4B (0.9 × 50 cm, 6–8 mg of lectin/mL of Sepharose) equilibrated in PBS. The mixture of [^{3}H]laminin oligosaccharides was applied to the GS I–Sepharose affinity column, and the unbound material was eluted with 2 column volumes of PBS buffer. The bound material was eluted with 2 column volumes of 50 mM Me-α-D-Gal. All lectin affinity chromatography was carried out at 4 °C. Fractions were analyzed as described above.

Exoglycosidase Treatment of Laminin Oligosaccharides and Gel Filtration Analysis. Purified Con A reactive oligosaccharides were digested with exoglycosidases, either sequentially or exhaustively, as indicated, and the reaction products were analyzed by gel filtration on a Bio-Gel P4 column (0.9 × 290 cm) eluted with 0.1 M acetic acid. Fractions (800 μL) were collected, and radioactivity was determined by scintillation counting in Atomlight scintillation fluid. Peaks were identified by their relative elution positions with respect to known oligosaccharide standards and purified isomaltodextrans.

Sequential digestion of the oligosaccharides was accomplished as follows: to a sample of oligosaccharide (10000 cpm) was added a single exoglycosidase (*C. perfringens* neuraminidase, 0.25 unit in 5 μL; New Castle disease virus neuraminidase, 0.05 unit in 5 μL; coffee bean α-galactosidase, 0.5 unit in 10 μL; *Aspergillus oryzae* β-galactosidase, 2.0 units in 20 μL; bovine testicular β-galactosidase, 18.5 milliunits in 5 μL; beef kidney β-*N*-acetylglucosaminidase, 0.5 unit in 25 μL; jack bean α-mannosidase, 1.25 units in 25 μL; bovine kidney α-L-fucosidase, 0.10 unit in 50 μL; or bovine epididymis α-L-fucosidase, 0.05 unit in 40 μL). Enzymolysis was allowed to proceed at 37 °C for 24 h in 100 mM $NaPO_4$ buffer, pH 6.0, followed by heating at 95 °C for 5 min prior to the addition of the next enzyme. This accomplished the sequential enzymatic degradation of the oligosaccharide, one residue at a time.

The exhaustive digestion of the oligosaccharide with multiple exoglycosidases was also carried out to allow the complete enzymatic degradation of oligosaccharide species containing repeating sequences in their peripheral structure. Exhaustive digestions were carried out as described for the sequential digestions, without the destruction of each enzyme.

Endoglycosidase Treatment of Laminin Oligosaccharides and Gel Filtration Analysis. Endoglycosidase digestions of purified laminin oligosaccharides were performed as previously described (Fukuda et al., 1984; Nakagawa et al., 1980). Briefly, 36 milliunits of endo-β-galactosidase from *E. freundii* in 50 μL of 100 mM $NaPO_4$ buffer, pH 6.0, was added to 250 μL containing 10000–20000 dpm of purified laminin oligosaccharides and incubated at 37 °C for 24 h. Reaction products were analyzed as described above. To ensure the complete degradation of the peripheral sugars of oligosaccharide chains, the addition of enzymes was repeated a second time.

Endo-β-galactosidase C digestion of laminin was carried out as described by Fushuku and co-workers (1987). Purified Con A reactive oligosaccharides were digested with 10 milliunits of endo C for 24 h at 37 °C. Oligosaccharides were analyzed before and after digestion for their ability to bind to GS I–Sepharose.

Acetolysis. Acetolysis was performed as described by Romero and Herscovics (1986). The dried acetolysis residue was dissolved in water and analyzed by gel filtration on a Bio-Gel P4 column (0.9 × 290 cm) eluted with 0.1 M acetic acid.

Results

Purification of Laminin from the Murine EHS Sarcoma. The α-D-Gal-specific lectin GS-I has been used as a rapid affinity chromatographic step for the purification of laminin from the EHS murine tumor (Shibata et al., 1982). The affinity-purified laminin contains four bands seen on SDS–PAGE. These include the 400- and 200-kDa subunits of laminin. In addition, we observed protein bands at apparent M_r 150 kDa (5–10%) and 80 kDa (0–5%), which were dissociated from the laminin subunits by heating in 0.1% SDS and analyzed on nonreducing PAGE, indicating that they were noncovalently associated with laminin. To accurately determine the abundance of the carbohydrate moieties of laminin, it was necessary to remove the two low molecular weight contaminants. Heating the samples in SDS followed by gel filtration on Sephacryl S-1000 allowed separation and recovery of all components, including laminin and the 150-kDa component. A representative elution profile is shown in Figure 1. The two high molecular weight bands, representing the subunits of laminin, eluted in peak 1, while the leading edge of peak 2 contained fractions enriched in the 150-kDa protein.

Identification of Laminin and the 150-kDa Band. The identity of the peaks was further established by the dot immunoblot assay. Anti-entactin antiserum reacted weakly with native laminin and strongly with entactin (peak 2) purified as described above. Anti-laminin antiserum reacted strongly with native laminin to which it was raised and only weakly with entactin (peak 2), which was present as a minor contaminant (<2%) of the immunogen (Figure 2).

As seen in Table I, the amino acid composition of laminin is in good agreement with literature reports, except for Lys and His (Timpl et al., 1979).

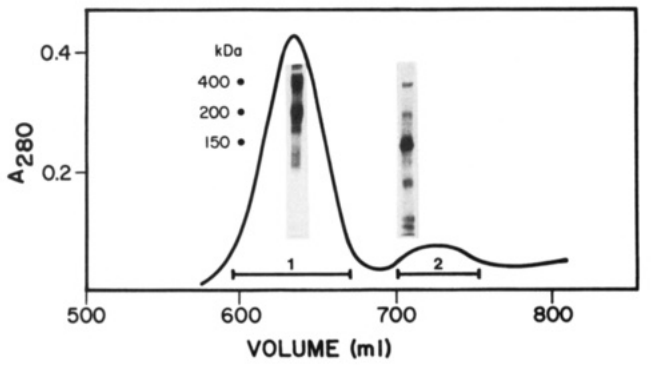


FIGURE 1: Sephacryl S-1000 gel filtration chromatography of laminin. Laminin purified by GS I–lectin affinity chromatography was prepared as described under Materials and Methods and applied to a column of Sephacryl S-1000 eluted in 0.6% LDS and 50 mM Tris, pH 7.2. Fractions (3 mL) were collected, and alternate fractions were analyzed by SDS–PAGE as shown.

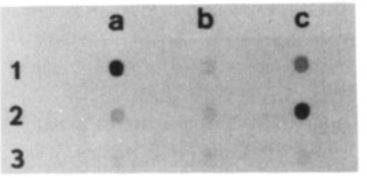


FIGURE 2: Dot immunoblot analysis of laminin and 150-kDa band. Antigens were (well 1) laminin, (well 2) 150-kDa band, and (well 3) BSA. Antigens in column a were reacted with anti-laminin antiserum. Antigens in column b were reacted with preimmune serum, and the antigens in column c were reacted with anti-entactin antiserum.

Table I: Amino Acid Analysis of Laminin[a]

residue	determined	lit. value[b]	residue	determined	lit. value[b]
Asp	115	109	Ile	40	42
The	53	58	Leu	101	92
Ser	76	77	Tyr	30	27
Glu	138	122	Phe	29	31
Pro	64	53	Lys	39	52
Gly	72	93	His	17	24
Ala	70	76	Arg	53	50
Val	47	48	Cys	ND[c]	30
Met	12	14	Trp	ND[c]	ND[c]

[a] Reported as residues per 1000 amino acid residues. [b] Literature values reported by Timpl et al. (1979). [c] ND, not determined.

Carbohydrate Analysis of Laminin and Entactin. Carbohydrate analysis (Table II) was performed on the purified laminin and entactin fractions. The results suggest that laminin is composed only of N-linked oligosaccharides due to the abundance of mannose and *N*-acetylglucosamine and the absence of GalNAc. In contrast, entactin appears to contain both N-linked oligosaccharides, as established by the presence of mannose, and O-linked oligosaccharides, as indicated by the presence of GalNAc.

Methylation Analysis. Methylation analysis of the total laminin oligosaccharides provides good evidence for the major oligosaccharide structures present. The results shown in Table III are consistent with a mixture of bi- and triantennary oligosaccharides (bi/tri, 45/65) that contain *N*-acetyllactosaminyl units (3Galβ1,4GlcNAc1β1).

The 2,4-di-*O*-methylmannose represents the mannose in β-linkage to the chitobiose core. The presence of 3,4,6-tri-*O*-methylmannose and 3,4-di-*O*-methylmannose indicates the presence of both bi- and triantennary structures. The absence of the 3,6-di-*O*-methylmannose precludes the possibility of a tetraantennary structure.

The presence of 2,4-di-*O*-methylgalactose indicates that we have a branched blood group I structure. The ratio of

Table II: Carbohydrate Analysis of Laminin and Entactin

carbohydrate	determined[a] laminin	determined[a] entactin
fucose	0.6	0.6
xylose	ND[b]	ND[b]
mannose	3.5	3.0
galactose	6.1	4.2
glucose	ND[b]	ND[b]
N-acetylgalactosamine	<0.05	1.03
N-acetylglucosamine	12.0	4.3
sialic acid	2.4	0.7
total	24.6	13.8

[a] Neutral carbohydrate as determined by phenol–sulfuric acid assay, 12.0%. Numbers represent grams of carbohydrate/100 g of protein. [b] ND, not determined.

Table III: Methylation[a] Analysis of Laminin Oligosaccharides

methylated monosaccharide	linkage	relative amount
2,3,4,6-tetra-*O*-methylgalactose	Gal1	2.6
2,4,6-tri-*O*-methylgalactose	3Gal1	4.3
2,4-di-*O*-methylgalactose	6)3Gal1	0.28
3,4,6-tri-*O*-methylmannose	2Man1	1.6
3,4-di-*O*-methylmannose	6)2Man1	0.6
2,4-di-*O*-methylmannose	6)3Man1	1.0
3,6-di-*O*-methylmannose	4)2Man1	ND[b]
3,6-di-*O*-methyl-2-acetamido-2-*N*-methyl-deoxyglucose	4GlcNAc1	4.0

[a] Methylation was performed as described under Materials and Methods. Methylated monosaccharides are identified as their alditol peracetates by gas chromatography. The molar amount of each residue is expressed relative to 1.0 residue of 2,4-di-*O*-methylmannose. [b] ND, not detected.

2,3,4,6-tetra-*O*-methylgalactose to 2,4,6-tri-*O*-methylgalactose to 3,6-di-*O*-methyl-GlcNAc is consistent with a predominance of structures Galα1,3Galβ1,4GlcNAc (i) and NeuNAcα2,3Galβ1,4GlcNAc (ii). It is clear, however, from the exoglycosidase experiments described below, that we can also establish the presence of the nonreducing terminal, Galβ1,4GlcNAc (iii), units among the Con A reactive oligosaccharides. The dominant peaks of 2,4,6-tri-*O*-methylgalactose and 3,6-di-*O*-methylglucosamine establish an abundance of poly(lactosamine) units: (3Galβ1,4GlcNAcβ1).

Specific Determination of Sialic Acid. The specific sialic acid present in laminin was determined as described under Materials and Methods. We established the presence of *N*-acetylneuraminic acid, but found no evidence for *N*-glycolylneuraminic acid, which is also reported to be present on murine glycoproteins (data not shown).

Analysis of the Terminal Nonreducing Residues of Laminin Oligosaccharides. We examined the oligosaccharides of intact laminin to determine the content of terminal α-Gal, β-Gal, and NeuNAc groups. The results are shown in Table IV. On the basis of the total carbohydrate composition of laminin (Table II) and the amount of galactose released by exoglycosidase digestion, we conclude that α-D-galactosyl units comprise 15.5–19.5% of the total galactose content of laminin, in good agreement with previous findings (Shibata et al., 1982). We also estimated that terminal nonreducing β-D-galactosyl units account for 3.5–4.5% of the total galactose content. NeuNAc comprises almost half of the terminal nonreducing residues.

Neuraminidase treatment did not increase the content of terminal α-D-galactosyl residues, indicating that the α-D-galactosyl units do not occur as penultimate residues on any oligosaccharide chains. Nor did we observe a significant (<10%) change in the content of NeuNAc following α-ga-

Table IV: Analysis of the Terminal Residues of Laminin[a]

treatment	% residue remaining		% residue exposed
	α-D-Gal[b]	NeuNAc	β-D-Gal
none	76.0 (100)	90.7 (100)	17.2 (100)
α-galactosidasae	7.2 (9)	82.1 (90)	26.8 (156)
neuraminidase	84.4 (111)	5.2 (6)	50.2 (292)
endo-β-galactosidase	31.2 (41)	62.1 (68)	11.5 (67)

[a] Intact laminin was treated as indicated and dialyzed extensively. The galactose dehydrogenase assay (see Materials and Methods) was used to determine the percentage of the terminal monosaccharide unit (α-D-Gal) remaining or the amount of penultimate monosaccharide residue exposed (β-D-Gal) following each enzymatic treatment. Similarly, the thiobarbituric acid assay was used to determine the quantity of NeuNAc remaining following each treatment. The specific content of each terminal monosaccharide prior to glycosidase treatment is defined as 100%. [b] Values are expressed in picomoles of monosaccharides per microgram of protein. The percentages indicate the proportion of monosaccharide remaining or exposed relative to the untreated molecules.

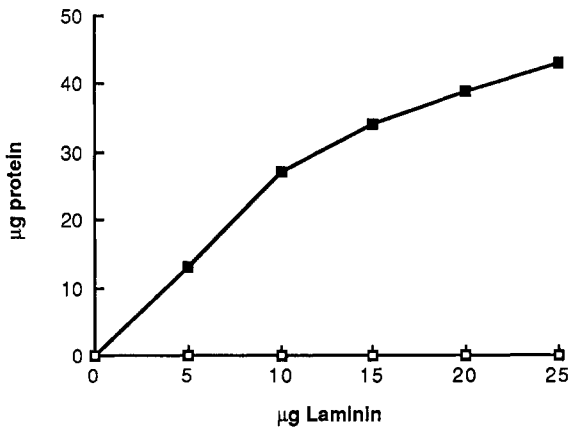


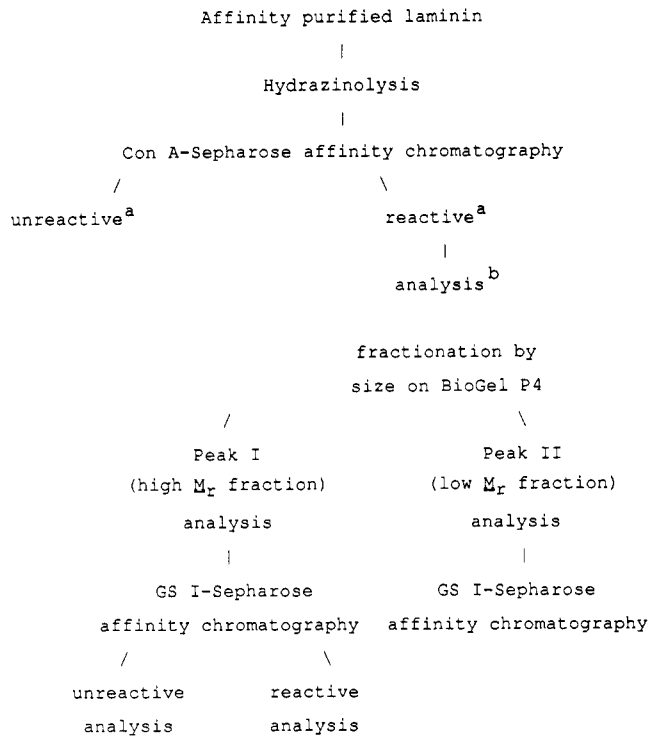
FIGURE 3: Precipitin reaction of laminin with the lectins from elderberry bark and from *M. amurensis* seeds. Laminin was incubated with either the lectin from *M. amurensis* (20 μg, solid boxes) or elderberry bark lectin (20 μg, open boxes); the precipitate was collected and assayed by the method of Lowry, as described under Materials and Methods.

lactosidase treatment, indicating that NeuNAc is not penultimate to terminal α-galactosyl end groups. However, β-D-galactosyl residues are penultimate to both NeuNAc and α-D-galactosyl units, as seen by the increase in β-galactosyl units following both α-galactosidase and neuraminidase digestion.

Endo-β-galactosidase liberates terminal di-, tri-, and tetrasaccharide structures from linear poly(lactosamine)-containing chains. The loss of terminal α-Gal, β-Gal, and NeuNAc groups, following treatment of laminin with endo-β-galacosidase, was indicative of the presence of poly(lactosamine) chains capped with these monosaccharide units. We found that 60% of the terminal α-D-galactosyl units, 30% of the terminal β-D-galactosyl units, and 30% of the NeuNAc units were released from the molecule by digestion with endo-β-galactosidase. Overall, 44% of the total terminal nonreducing monosaccharide residues were removed by endo-β-galactosidase.

To establish the specific linkage of sialic acid to the penultimate galactosyl residue, we used several approaches. The lectin from elderberry bark, which shows a specificity for NeuNAcα2,6Gal/GalNAc (Shibuya et al., 1987), did not form a precipitate with laminin, whereas the *M. amurensis* lectin, recently described by Wang and Cummings (1988) and which is specific for NeuNAcα2,3lactose, reacted strongly with laminin (Figure 3); it was shown to be specific for NeuNAcα2,3lactose by hapten inhibition. NeuNAcα2,6-

Scheme I



[a] Reactive and unreactive denote binding to the indicated lectin affinity resin. [b] Analysis denotes endo- and exoglycosidase digestion of the radiolabeled oligosaccharides followed by gel filtration chromatography of the reaction products on Bio-Gel P4, as described under Materials and Methods.

lactose did not inhibit precipitation of laminin by this lectin. Further, the precipitin reaction was inhibited by neuraminidase treatment (data not shown). We also treated the Con A reactive oligosaccharides of laminin exhaustively with New Castle disease virus neuraminidase (described below), which is specific for NeuNAcα2,3Gal. The NeuNAc content of laminin was completely released by this enzyme. Finally, the absence of both 2,3,6-tri-*O*-methylgalactose and 2,3,4-tri-*O*-methylgalactose in methylation analysis shows that terminal NeuNAcα2,4Gal and NeuNAcα2,6Gal units, respectively, are absent.

To ascertain the linkage of the terminal α-galactosyl groups, we used the enzyme endo-β-galactosidase C (endo C) recently described by Fushuku et al. (1987), which was reported to be specific for the terminal nonreducing disaccharide Galα1,3Galβ1,4, cleaving the β1,4 bond. This enzyme does not act as an exo-α-galactosidase. Con A reactive oligosaccharides of laminin were analyzed by chromatography on GS I–Sepharose before and after treatment with endo C. While 70% of the untreated oligosaccharides bound to the affinity column, less than 5% of the endo C treated material was bound and specifically eluted with Me-α-D-Gal. Additionally, as stated above, the failure to detect either 2,3,4-tri-*O*-methylgalactose or 2,3,6-tri-*O*-methylgalactose militates against the presence of either Galα1,6Gal or Galα1,4Gal.

Experimental Approach. The strategy employed for the elucidation of the structure of the oligosaccharide units of laminin is outlined in Scheme I.

Hydrazinolysis Release of Oligosaccharides and Fractionation by Lectin Affinity Chromatography. The N-linked glycans were released from laminin by hydrazinolysis, as described under Materials and Methods. Starting materials and reaction products were analyzed for neutral and amino sugars. Yields calculated on the basis of these analyses typically

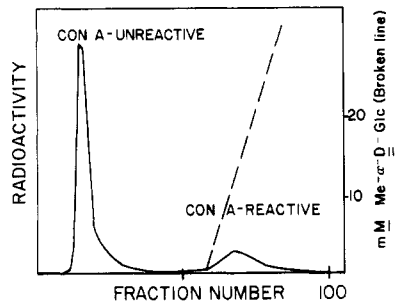


FIGURE 4: Binding of laminin oligosaccharides to Con A–Sepharose. Hydrazinolysis-released, NaB^3H_4 reduced laminin oligosaccharides were applied to a column of Con A–Sepharose in PBS. The column was washed with 4 column volumes of buffer, and bound oligosaccharides were eluted with a linear gradient of 0–50 mM Me-α-D-Glc*p* (broken line), followed by 10 column volumes of Me-α-D-Man in PBS (not shown). Oligosaccharides recovered in the buffer wash were designated Con A unreactive; those eluting with haptenic sugar were designated Con A reactive.

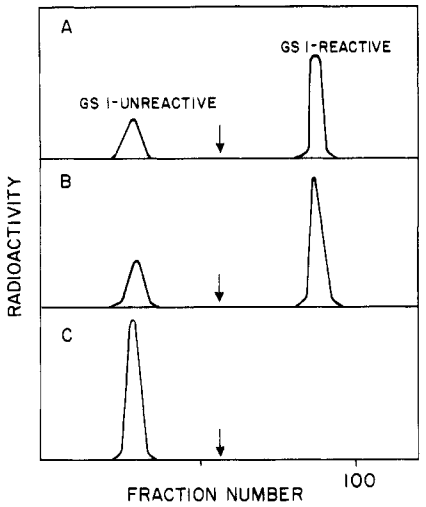


FIGURE 5: Binding of Con A reactive laminin oligosaccharides to GS I–Sepharose. Con A reactive oligosaccharides from Figure 4 were applied to a column of GS I–Sepharose (0.9 × 45 cm) in PBS. The column was washed with 2 column volumes of buffer, and bound oligosaccharides were eluted with 2 column volumes of Me-α-D-Gal in PBS (arrow). Fractions (1 mL) were collected and analyzed by scintillation counting. Specificity of binding was demonstrated by exoglycosidase digestion: (A) untreated oligosaccharides; (B) oligosaccharides treated with neuraminidase prior to GS I affinity chromatography; (C) oligosaccharides treated with neuraminidase and α-galactosidase prior to GS I affinity chromatography.

demonstrated 60–65% recovery of oligosaccharides. The free, radiolabeled oligosaccharides were analyzed by lectin affinity chromatography, endo- and exoglycosidase digestion followed by gel filtration analysis, acetolysis, and methylation analysis. Within the limitations of the 65% recovery of oligosaccharides following hydrazinolysis, conclusions were made regarding the structures of the laminin oligosaccharides.

The laminin oligosaccharides were first fractionated on Con A–Sepharose as shown in Figure 4. All of the bound material eluted as a single peak with a linear gradient of 0–50 mM Me α-D-Glc. The Con A reactive (Figure 5) oligosaccharides were applied to a column of GS I–Sepharose, and bound oligosaccharides were eluted with 100 mM Me α-D-Gal. To demonstrate specificity of the interaction with the GS I–Sepharose column, the oligosaccharides were digested with either neuraminidase (panel B), which had no effect on the binding of the oligosaccharide to GS I–Sepharose, or α-galactosidase (panel C), which abolished binding of the oligosaccharide to GS I–Sepharose. Seventy percent of the Con A reactive oligosaccharides were bound and specifically eluted from the GS I–Sepharose column (Figure 5A).

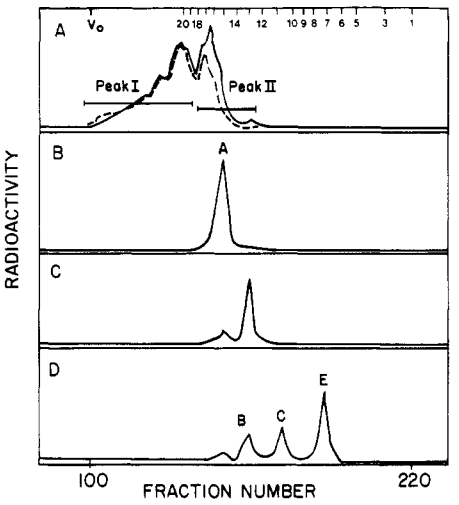


FIGURE 6: Gel filtration chromatography of the sequential digestion of Con A reactive peak II. The Con A reactive peak II oligosaccharides were sequentially digested with exoglycosidases and analyzed as described under Materials and Methods: (A) total Con A reactive oligosaccharides, untreated (broken line) and neuraminidase treated (solid line); (B) the peak II, neuraminidase-digested, reaction products from panel A were treated with α-galactosidase; (C) the reaction products from panel B were treated with β-galactosidase; (D) the reaction products from panel C were treated with *N*-acetyl-β-glucosaminidase. Letters indicate peaks referred to in the text.

Table V

digestion with[a]	reaction 1	2	3	4	5	6
α-galactosidase	−	+	−	+	−	+
neuraminidase[b]	−	−	+	+	+	+
β-galactosidase	+	+	+	+	+	+
β-*N*-acetylglucosaminidase	+	+	+	+	+	+
% $Man_3GlcNAc_2$–OH product	18	28	32	72	0	72

[a] Con A reactive oligosaccharides were digested exhaustively (reactions 1–4, 6) or sequentially (reaction 5) with the indicated exoglycosidases. Reaction products were analyzed by gel filtration, and the percentage of radioactivity eluting as $Man_3GlcNAc_2$–OH was determined. [b] *C. perfringens* neuraminidase was used in reactions 3–5. New Castle disease virus neuraminidase was used in reaction 6.

The size distribution of the Con A reactive oligosaccharides was determined by gel filtration chromatography on a Bio-Gel P4 column, equilibrated with 100 mM acetic acid. We observed a broad distribution of molecular sizes eluting as several overlapping peaks. This elution profile is shown in panel A of Figure 6. Two peaks of radioactivity were pooled as indicated in panel A of this figure and referred to as Con A reactive peak I and peak II.

Analysis of the Terminal Nonreducing Residues of the Con A Reactive Oligosaccharides. The total Con A reactive oligosaccharide fraction was further analyzed to determine the degree to which oligosaccharides are substituted with terminal nonreducing α-D-Gal, β-D-Gal, and sialic acid groups. The results of these experiments are shown in Table V. The exhaustive digestion of the oligosaccharide sample with a mixture of β-galactosidase and *N*-acetyl-β-glucosaminidase (reaction 1) converted 18% of the total oligosaccharides in this mixture to $Man_3GlcNAc_2$–OH. Therefore, we deduce that 18% of the Con A reactive oligosaccharides contain the minimal terminal structure Galβ1,4GlcNAcβ1 (iv). When α-galactosidase was included in the digestion, 28% of the oligosaccharides were converted to $Man_3GlcNAc_2$–OH (reaction

2). Therefore, we conclude that 10% of the oligosaccharides contain at least one branch with the terminal structure Galα1, Galβ1,4GlcNAcβ1 (v), and the second branch would have a structure similar to iv or v. Following digestion of the oligosaccharide mixture (reaction 3) with neuraminidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase, 32% of the oligosaccharides were converted to a peak that coeluted with $Man_3GlcNAc_2$–OH. These results indicate that 14% of the oligosaccharides contain at least one branch with the terminal structure NeuNAcα2, Galβ1,4GlcNAcβ1 (vi). The second branch may contain either structure iv or vi, but not structure v, since omission of α-galactosidase prevents degradation of these structures to $Man_3GlcNAc_2$–OH.

The oligosaccharide mixture was digested exhaustively with neuraminidase, α- and β-galactosidase, and *N*-acetyl-β-glucosaminidase (reaction 4), resulting in 72% of the oligosaccharide mixture coeluting with $Man_3GlcNAc_2$–OH. Therefore, 30% of the oligosaccharides must have one branch containing structure v and a second branch containing structure vi, since both neuraminidase and α-galactosidase were required to digest 30% of these structures to the peak eluting as $Man_3GlcNAc_2$–OH. Therefore, 40% of the oligosaccharides of laminin contain at least one terminal α-galactosyl groups (10% have α-Gal only, 30% contain both α-Gal and NeuNAc). Of the remaining oligosaccharides present in the mixture, 32% do not contain terminal α-galactosyl residues (the omission of α-galactosidase still allows the degradation of these structures to the $Man_3GlcNAc_2$–OH peak).

Twenty-eight percent of the laminin oligosaccharides are not converted to $Man_3GlcNAc_2$–OH by exhaustive digestion with the exoglycosidases described above (neuraminidase, α- and β-galactosidase, β-*N*-acetylglucosaminidase). Additional peripheral carbohydrate residues, such as α-L-fucose, may be present that block the exoglycosidase reactions and produce reaction products larger than $Man_3GlcNAc_2$–OH. This question will be examined below.

In Table V, reaction 5, the Con A reactive oligosaccharides have been sequentially digested with neuraminidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase to degrade each branch by a single lactosamine unit. Omission of α-galactosidase (reaction 5) failed to produce a peak corresponding to $Man_3GlcNAc_2$–OH. This implies that all structures containing a single *N*-acetyllactosamine unit on each branch must contain at least one terminal α-D-Gal unit.

Peak II Oligosaccharides. Sequential exoglycosidase digestion of Con A reactive peak II is shown in Figure 6. In panel A, the untreated material eluted as multiple broad peaks (broken line). Removal of sialic acid produced a slight shift in the elution pattern to lower molecular weight (panel A, solid line). As we can see in the progress from panel A to panel B, digestion of peak II material with α-galactosidase yielded a narrower symmetric peak (A), indicating that much of the heterogeneity of peak II is due to differences in substitution by α-D-galactosyl residues. Further exoglycosidase degradation of the major peak by β-galactosidase results in the removal of two β-D-galactosyl units and a shift of the peak by two glucose units as shown in panel C; treatment of the major peak with β-hexosaminidase resulted in the removal of two *N*-acetyl-β-glucosaminyl units and a shift in the peak by four glucose units, as shown in panel D. The resulting peak (E) in panel D migrated with a mobility of 7.5 glucose units compared to the dextran oligosaccharide standards, which is in good agreement with the elution position of standard $Man_3GlcNAc_2$–OH (Yamashita et al., 1982). Furthermore, digestion of this peak with α-mannosidase generated a peak

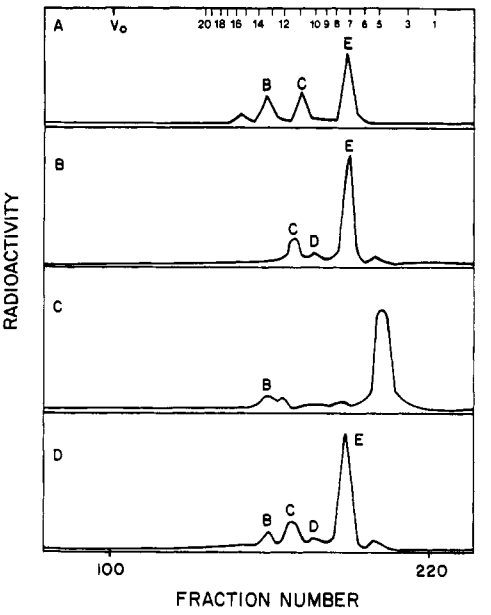


FIGURE 7: Gel filtration chromatography of Con A reactive oligosaccharide sensitive to α-fucosidase and α-mannosidase. The Con A reactive oligosaccharides from Figure 6D eluting at a larger size than $Man_3GlcNAc_2$ were treated as follows: (A) the Con A reactive peak II (Figure 6A) was treated as described for panel D; (B) the product from panel A was treated with α-L-fucosidase and *N*-acetyl-β-glucosaminidase; (C) the product from panel A was treated with α-mannosidase; (D) for comparison the total Con A reactive oligosaccharides were exhaustively digested with neuraminidase, α- and β-galactosidase, and hexosaminidase. Letters indicate peaks referred to in the text.

migrating at 5.5 glucose units (not shown) that coeluted with the known standard Manβ1,4GlcNAcβ1,4GlcNAc–OH. Therefore, we propose the following partial structure for the major component of Con A reactive peak II (structures VIII–X of Table VI):

$$\begin{array}{lll} & [\ \ ]\ \text{Gal}\beta1,4\text{GlcNAc}\beta1,2\text{Man}\alpha1,3/6 & \\ \text{NeuNAc}\alpha2,3 & & \text{Man}\beta1,\ \text{GlcNAc}_2 \quad \text{(A)} \\ \text{Gal}\alpha1,3 & [\ \ ]\ \text{Gal}\beta1,4\text{GlcNAc}\beta1,2\text{Man}\alpha1,6/3 & \end{array}$$

The percentage abundance of these structures is estimated from the following information: (1) peak II represents 40% of the total Con A reactive oligosaccharides; (2) 50% of the peak II oligosaccharides cannot be degraded to $Man_3GlcNAc_2$–OH, even with exhaustive digestion; (3) omission of α-galactosidase (Table V, reaction 5) precludes degradation of the Con A reactive oligosaccharides to $Man_3GlcNAc_2$–OH. We conclude from point 3 that all structures of the form A will contain at least one α-D-galactosyl residue; therefore, structure X does not exist. From points 1 and 2 we can conclude that the structures containing sialic acid and one α-D-galactosyl residue (structure IX) and those structures containing only α-D-galactosyl residues (structure VIII) must represent 20% of the total Con A reactive oligosaccharides. The abundance of each structure reported in Table VI has been estimated in a similar fashion.

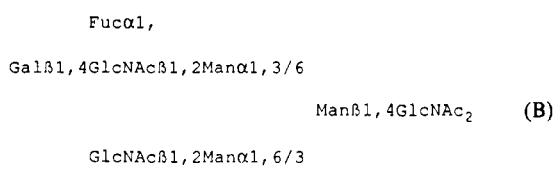
In Figure 6D, three peaks (B–D) corresponding to approximately 20% of the Con A reactive oligosaccharides were not converted to the $Man_3GlcNAc_2$–OH core by sequential exoglycosidase digestion. This was not due to incomplete digestion inasmuch as (1) the elution profiles were reproducible and (2) exhaustive exoglycosidase digestion (Table V) gave the identical results, indicating that their resistance might be due to the presence of additional nonreducing monosaccharides, such as L-Fuc or D-Man. To test this hypothesis,

Table VI: Structures of the Asparagine-Linked Oligosaccharides of Laminin

	Nonreducing terminus	Structure	Fig/Panel/Peak	%Abundance
I)	NeuNAcα2,3]0,1,2	Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4(GlcNAcß1,3Galß1)$_m$4GlcNAcß1,2Manα1,6/3	8/C/J	3%
II)	Galα1,3]1,2	Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4(GlcNAcß1,3Galß1)$_m$4GlcNAcß1,2Manα1,6/3	8/A/J	3%
III)	NeuNAcα2,3]0,1,	Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	8/C/H	5%
IV)	NeuNAcα2,3]1 Galα1,3]1	Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galα1,3Galß1,4GlcNAcß1,2Manα1,6/3	8/A/G	15%
V)	Galα1,3]1,2	Galß1,4GlcNAcß1,6 Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Neuα2,3Galß1,4GlcNAcß1,2Manα1,6/3	8/B/K	13%
VI)	NeuNAcα2,3]0,1,2,3	Galß1,4GlcNAcß1,6 Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4(GlcNAcß1,3Galß1)$_m$4GlcNAcß1,2Manα1,6/3	8/C/D	12%
VII)	NeuNAcα2,3]0,1,2 Galα1,3]1,2,3	Galß1,4GlcNAcß1,6 Galß1,4(GlcNAcß1,3Galß1)$_n$4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4(GlcNAcß1,3Galß1)$_m$4GlcNAcß1,2Manα1,6/3	8/A/F	12%
VIII)	Galα1,3]1,2	Galß1,4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	5/D/E	\
IX)		Galα1,3Galß1,4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ NeuNAcα2,3Galß1,4GlcNAcß1,2Manα1,6/3	5/D/E	/ 20%
X)	NeuNAcα2,3]0,1,2	Galß1,4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	5/D/-	0%
XI)		Fucα1,\ Galß1,4GlcNAcß1,2Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	6/C/B	8%
XII)		Manα$_3$1, Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	6/A/D	3%
XIII)		Manα$_4$1, Manα1,3/6 Manß1,4GlcNAc$_2$ Galß1,4GlcNAcß1,2Manα1,6/3	6/A/C	6%

the exhaustively digested oligosaccharide mixture, shown in Figure 7D, was incubated with either α-L-fucosidase or α-mannosidase. Figure 7B shows the products of digestion with α-L-fucosidase and *N*-acetyl-β-glucosaminidase. Peak B, shown in panel A, was converted to $Man_3GlcNAc_2$–OH following treatment with α-L-fucosidase and *N*-acetyl-β-glucosaminidase, implying that it contains a Fucα1,-GlcNAc unit attached to the trimannosyl core. The sensitivity of peak B to α-L-fucosidase indicated the presence of at least one fucose residue; however, from the elution position of the molecule it is unclear whether there are one or two fucose residues present on the oligosaccharide.

The species in peak B is too large to be $Man_3GlcNAc$-(Fuc)GlcNAc–OH. Furthermore, peak B was insensitive to digestion with α-mannosidase, indicating that peripheral residues are present. The sensitivity of this peak to α-L-fucosidase and *N*-acetyl-β-glucosaminidase and its apparent size suggest that peak B has the structure (Table VI, structure XI)

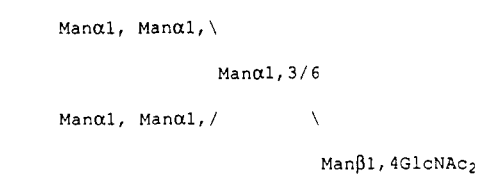


(B)

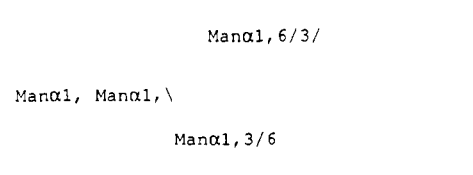
Since the peak B species shown in Figure 7 appears as a consequence of digestion with neuraminidase, α-galactosidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase, we propose that these oligosaccharides contain at least one branch of the form [ ] Galβ1,4GlcNAc, where [ ] indicates a possible substitution with Galα1, or NeuNAcα2, at the nonreducing terminus of the oligosaccharide. This oligosaccharide is similar to one reported by Yamashita and co-workers (Yamashita et al., 1980). We would anticipate that this structure was produced by exoglycosidase action on the oligosaccharide shown in Table VI, structure XI. Yamashita and co-workers (Yamashita et al., 1980) reported that jack bean β-galactosidase is inactive toward the Galβ1,4GlcNAc bond when the penultimate GlcNAc is substituted with fucose. We do not know if fucose similarly inhibits the bovine testicular and *Aspergillus* β-galactosidases. However, if fucose acts as an inhibitor for these enzymes, this suggests that after β-galactosidase digestion we might have a terminal Galβ1 remaining on the fucose containing chain. This oligosaccharide structure will require further confirmation.

To determine if peaks C and D are hybrid structures, we digested the reaction products shown in Figure 7A with α-mannosidase. In this case, sensitivity to α-mannosidase was indicative of hybrid and not high mannose structures, because peaks C and D in Figure 7 appear only after digestion with β-galactosidase and *N*-acetyl-β-glucosaminidase. Peaks C and D were eliminated (panel C) following α-mannosidase digestion of the oligosaccharide mixture, demonstrating the presence of terminal Manα1, units. In addition, peak E, the $Man_3GlcNAc_2$–OH core, was further degraded by α-mannosidase to $Man_1GlcNAc_2$–OH, as expected. Due to the contaminating activity of β-mannosidase in *N*-acetyl-β-glucosaminidase, this core was further degraded to $GlcNAc_2$–OH, eluting at approximately 4.5 glucose units.

The hybrid structures found in peak II do not appear to contain poly(lactosamino) repeating units, because (1) the low molecular size indicated for the peak II molecules as shown in Figure 6 precludes the presence of a poly(lactosamino) structure, (2) a single round of sequential exoglycosidase digestion degraded the peripheral Galβ1,4GlcNAc structure, and (3) all the hybrid molecules found in the Con A reactive peak II oligosaccharides accounted for all hybrid species observed in the total Con A reactive material. Peak C elutes at a position greater than 10 glucose units, which makes identification tentative. Peak D elutes at the position of 10 glucose units. On the basis of the estimated size determined by their elution position from the Bio-Gel P4 column, the following hybrid structures are proposed as the species in peaks C and D (Table VI, structures XII and XIII):



(C)



(D)

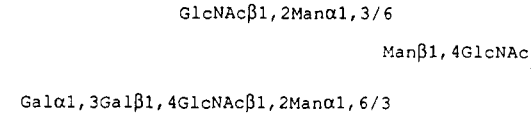
The structure proposed for peak D, eluting at 10 glucose units, is in good agreement with the elution Yamashita and co-workers (Yamashita et al., 1982) reported for this structure. These structures would have arisen from the structures shown in Table VI, structures XII and XIII.

Peak I Oligosaccharides. In contrast to the Con A reactive peak II oligosaccharides, the larger peak I oligosaccharides were not significantly degraded by the sequential glycosidase digestion under conditions sufficiently rigorous to remove only a single *N*-acetyllactosamine unit from each branch. This single round of exoglycosidase digestion did not convert any oligosaccharides of peak I into peaks corresponding to a known structure (e.g., $Man_3GlcNAc_2$–OH) (data not shown). Comparing experiments in which two Galβ1,4GlcNAc units were digested from each branch with experiments in which only a single Galβ1,4GlcNAc unit was removed, we observed that the proportion of $Man_3GlcNAc_2$–OH in the products was virtually unchanged (data not shown). This result is consistent with the proposal that peak I contains poly(lactosaminyl) structures. This would also indicate that at least one branch of the trimannosyl core of these poly(lactosamine)-containing oligosaccharides has the minimum structure

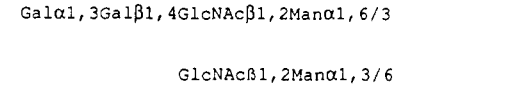
[ ] $(Gal\beta1,4GlcNAc\beta1,3)_2$ Galβ1,4GlcNAcβ1,2Manα1

To test this possibility directly, endo-β-galactosidase digestion, alone and in combination with exoglycosidases, was used to further probe the structures of the peak I oligosaccharides. The results are shown in Figure 8.

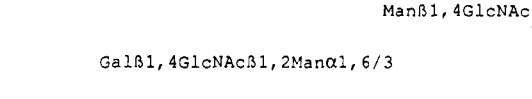
We propose that the three peaks labeled G, H, and J, generated by the action of endo-β-galactosidase, represent the products



(G)



(H)



(J)

This hypothesis was tested by digesting with endo-β-galactosidase as well as the exoglycosidases indicated in Figure 8C,D. Peak G is converted to peak H by α-galactosidase (panel C), and peak H is converted to peak J by β-galacto-

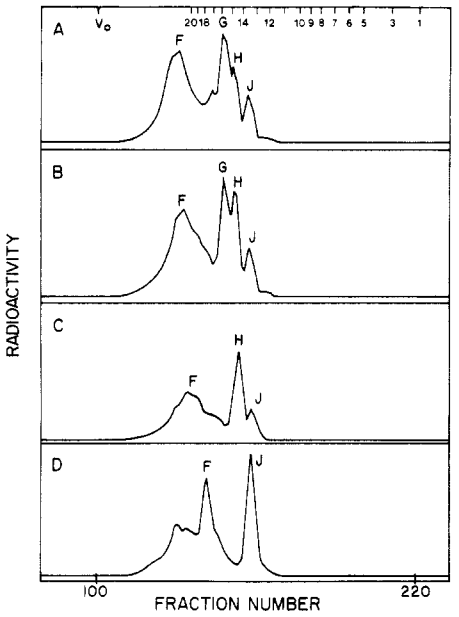


FIGURE 8: Gel filtration chromatography of Con A reactive peak I treated with endo-β-galactosidase. The Con A reactive peak I oligosaccharides were examined for their sensitivity to endo-β-galactosidase alone and in combination with exoglycosidases. Reaction products were analyzed as described in the legend to Figure 6. (A) Peak I oligosaccharides were treated with endo-β-galactosidase; (B) the reaction products from panel A were treated with neuraminidase; (C) the reaction products from panel A were treated with α-galactosidase; (D) the reaction products from panel C were treated with β-galactosidase. Letters indicate peaks referred to in the text.

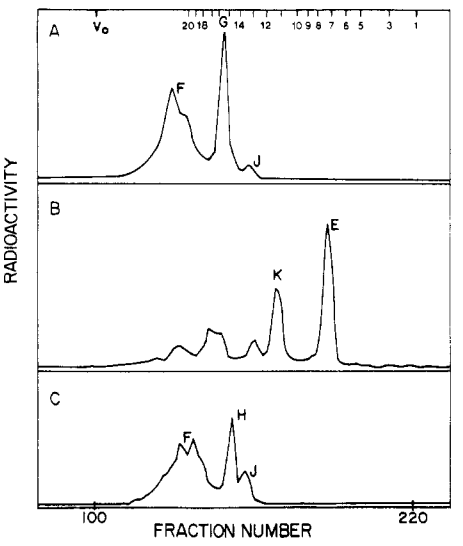


FIGURE 9: Gel filtration chromatography of Con A reactive–peak I GS I reactive and unreactive oligosaccharides treated with endo-β-galactosidase. The Con A reactive peak I GS I reactive oligosaccharides were digested by endo- and exoglycosidases (indicated below) and analyzed as described in the legend to Figure 6. (A) Con A reactive–peak I GS I reactive oligosaccharides were treated with endo-β-galactosidase; (B) Con A reactive–peak I GS I reactive oligosaccharides were treated exhaustively with α-galactosidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase; (C) Con A reactive–peak I GS I unreactive oligosaccharides were treated with endo-β-galactosidase. Letters indicate peaks referred to in the text.

sidase (panel D). It was also possible to convert peak J to the $Man_3GlcNAc_2$–OH core by using *N*-acetyl-β-glucosaminidase (data not shown). The specificity of endo-β-galactosidase dictates that a terminal nonreducing GlcNAc residue remains following enzymatic cleavage of poly(*N*-acetyllactosamine) oligosaccharides. We also know that endo-β-galactosidase does not act as an exo-β-galactosidase. Therefore, peak G would have arisen from structure IV, peak H from structure III, and peak J from both structures I and II, as shown in Table VI, where *n* and *m* indicate that there exists a family of oligosaccharides that vary in the number of lactosamine repeating units in each branch. In most cases n and $m \geq 2$, since two rounds of sequential digestion did not result in an increase in the amount of $Man_3GlcNAc_2$ over that observed for a single round of exoglycosidase digestions (data not shown).

It is important to note here that there does not appear to be any evidence for the existence of a structure similar to structure G substituted with NeuNAc on the short arm since digestion with α-galactosidase, β-galactosidase, and endo-β-galactosidase did not reveal an undigested peak in panel D. We are unable to explain the apparent change in the elution profile following neuraminidase digestion. However, this change alone is not sufficient evidence on which to propose an additional oligosaccharide species.

To examine the distribution of α-D-galactosyl units on poly(lactosamine)-containing structures, the peak I oligosaccharides were fractionated on GS I–Sepharose: 75% was specifically bound; 25% was not bound to the column. Con A reactive peak I, GS I reactive material was digested with endo-β-galactosidase, as shown in Figure 9A. We observed that peaks F and G are the predominant peaks with a small amount of peak J present, indicating that structure H contains no α-D-galactosyl terminal residues, while structure J contains at least a small proportion of terminal α-D-galactosyl residues. It is not possible from these data to determine the number of terminal nonreducing α-D-galactosyl units on structures F, G, or J. This represents a probable source of microheterogeneity in these structures.

In Figure 9C the Con A reactive, GS I unreactive oligosaccharides were digested with endo-β-galactosidase. The resulting pattern demonstrates that structure G, containing terminal α-Gal, was removed as predicted and that structure H contains no terminal α-galactosyl groups. We also observed that there exists a population of structure J that does not contain terminal α-galactosyl residues. Additionally, the presence of peak F indicates heterogeneity in terminal monosaccharide residues of the proposed blood group I structures.

Occurrence of the Blood Group I Structure. Following endo-β-galactosidase digestion, a large peak (40% of the peak I oligosaccharides) of undigested material was eluted at the same position as peak I (Figures 8 and 9). The structure of the undigested peak F is deduced from three lines of evidence found in Figures 7 and 8: (1) The size of the oligosaccharides indicates that they are larger than the biantennary structures found in peak II, containing a single Galβ1,4GlcNAc unit on each branch. (2) The continuous digestion of the Con A reactive oligosaccharides with α-galactosidase, neuraminidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase (Figure 7D) destroyed peak F, suggesting that the structure contained only the (Galβ1,4GlcNAc) repeating unit. However, the resistance of peak F to endo-β-galactosidase indicates that the poly(lactosamine) chain is not a simple linear structure. (3) The presence of 2,4-di-*O*-methylgalactose found in methylation analysis supports the presence of a branched structure, which would be insensitive to endo-β-galactosidase. We therefore propose that peak F has the structure shown in Table VI, structure VII.

Further examination of Figure 9B shows the result of the exhaustive exoglycosidase digestion of the Con A reactive peak I GS I reactive material with α-galactosidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase. This combination of enzymes should degrade all structures that do not contain a terminal NeuNAcα2 residue to $Man_3GlcNAc_2$–OH. The structure in

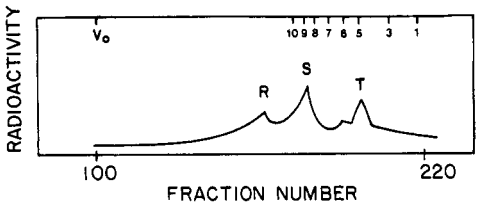


FIGURE 10: Gel filtration chromatography of Con A reactive oligosaccharides subject to acetolysis. Con A reactive oligosaccharides were subjected to acetolysis as described under Materials and Methods. Reaction products were analyzed as described in the legend for Figure 6. Letters indicate peaks referred to in the text.

peak E is $Man_3GlcNAc_2$–OH, as shown in Figure 7B by its sensitivity to α-mannosidase and its coelution at 7.5 glucose units with the standard structure. We deduce the structure of peak K as follows: (1) Peak K elutes at a position of approximately 11 glucose units. (2) Peak K appears when neuraminidase is omitted from the exhaustive digestion of this oligosaccharide mixture and therefore must contain a terminal NeuNAcα2 unit. (3) The exhaustive digestion of peak I mixture with α-galactosidase, β-galactosidase, β-hexosaminidase, and neuraminidase will completely degrade these oligosaccharides to $Man_3GlcNAc_2$–OH. (4) Those oligosaccharides that have a single linear poly(lactosamine) chain on one arm do not have NeuNAc on the short arm, as discussed above. On the basis of its size suggested by its elution position, we propose that it contains the structure

NeuNAcα2,3Galβ1,4GlcNAcβ1,2Man (K)

on one arm, while the other arm contains the blood group I structure. In Figure 9A this oligosaccharide species is concealed among the structures undigested in peak F. Thus, peak K would represent the structure

Manα1,3/6\

Manβ1, $GlcNAc_2$ (K)

NeuNAcα2,3Galβ1,4GlcNAcβ1,2Manα1,6/3/

and could have originated from structure V of Table VI. We propose that structure K is one of a family of blood group I structures in the Con A reactive oligosaccharides.

Acetolysis. The results of the acetolysis of Con A reactive oligosaccharides reductively labeled in the reducing GlcNAc are shown in Figure 10. Three broad peaks were obtained that eluted at positions labeled R–T. Peak R, on the basis of the size estimated by its elution position, may correspond to the structure

Manß1,4GlcNAc$_2$-O[^{3}H]

Galα1,3](Galß1,4GlcNAcß1,3)$_2$ Manα1,3/ (R)

NeuNAcα2,3]

Peak S, the predominant structure, elutes at approximately 10.5 glucose units. We propose that this corresponds to

Manß1,4GlcNAc$_2$-O[^{3}H]

Galα1,3]Galß1,4GlcNAcß1,2Manα1,3/ (S)

NeuNAcα2,3]

Peak T eluted at the lowest molecular size, corresponding to 6.5 glucose units. We propose that this peak corresponds to

Manβ1,4GlcNAc$_2$-O[^{3}H]

Manα1,3/ (T)

The abundance of peak S suggests that the Manα1,6 branch contains much of the poly(lactosamine) structure observed. Structure T is apparently a degradation product of the acetolysis reaction resulting from the loss of the structure on the Manα1,3 branch.

Discussion

The glycosyl chains of laminin provide one of the most heterogeneous arrays of oligosaccharides reported for any glycoprotein thus far. In fact, the extent of heterogeneity made it impossible to isolate a single unique molecular species. We therefore chose to fractionate the hydrazinolysis-released oligosaccharides into groups on the basis of their ability to bind to immobilized lectins, namely, Con A–Sepharose and GS I–Sepharose. Subsequently, sequential degradation of the oligosaccharides with purified endo- and exoglycosidases converted a complicated mixture to a common product, Manβ1,4GlcNAcβ1,4GlcNAc–OH, and permitted the deduction of the peripheral structure of the original family of oligosaccharides.

During the course of our work some investigators (Chung et al., 1979; Arumugham et al., 1986) reported only 12–15% carbohydrate on laminin, while our results indicate that laminin contains 25–27% carbohydrate by weight. The difference between our results and those reported by Chung may be due to different sources of laminin. However, Arumugham and co-workers characterized EHS laminin, and our differences are not easily explained. Arumugham and co-workers reported both high mannose and tetraantennary oligosaccharides, for which we have no evidence. The difference in our results may be due to the differences in purification techniques, which would yield unique subpopulations of laminin. Because we are isolating only laminin that contains the terminal α-D-Gal residue, we are probably isolating a greater proportion of mature laminin forms. Arumugham et al. (1986) observed high mannose oligosaccharides in their laminin preparation, consistent with the possibility that they were working with a mixture of precursor and mature forms. The original purification reported by Timpl yielded about 0.5 mg of laminin/g of the EHS tumor. The lectin affinity chromatography step that we have employed yields 6.0 mg of laminin/g of tumor. Kleinman and co-workers (Kleinman et al., 1982) reported extracting 16 mg of laminin/g of EHS tumor using 2 M guanidine hydrochloride. We have not been able to extract all the laminin present in the EHS tumor; however, our yields of laminin are at least 10-fold greater than those obtained by Timpl and co-workers (Timpl et al., 1979). For this reason we believe our results are more representative of the oligosaccharide moieties of murine laminin than those reported by Arumugham and co-workers.

We have elucidated a number of interesting and possibly unique features of the Con A reactive oligosaccharides of laminin. First, as shown by their sensitivity to endo-β-galactosidase, the Con A reactive oligosaccharides contain a poly(lactosamino) structure, a rare occurrence for biantennary chains (Fukuda et al., 1984). These oligosaccharides also contain the terminal nonreducing α-D-Gal*p* residue as observed in other murine glycoconjugates (Eckhart & Goldstein, 1983a,b; Maddox et al., 1982; Petryniak et al., 1986; Mohan & Spiro, 1986) in addition to the thyroglobulins of several nonprimate species (Spiro & Bhoyroo, 1984).

We presented evidence that the blood group I determinant occurs as a component of a large proportion (40%) of the Con A reactive laminin oligosaccharides as evidenced by the resistance of some of the larger oligosaccharides to digestion with endo-β-galactosidase and sensitivity to sequential exoglycosidase digestion. The derivative 2,4-dimethylgalactose expected from the blood group I structure, identified by gas

chromatography by coelution with a known standard, represented approximately 4% of the total galactose. Due to the larger size and complexity of the Con A unreactive oligosaccharides, we have not yet been able to accurately determine the amount of blood group I structure in the Con A unreactive material. If we assume that this structure is present on both the Con A reactive and unreactive oligosaccharides in similar proportions, then it represents approximately 40% of the oligosaccharide structures of laminin. If we accept that there are 7 galactose residues per oligosaccharide (6.9 residues of the tri- and tetramethylgalactose per 2,4-dimethylmannose) and make the further assumption that we require only a single blood group I structure per oligosaccharide to make it endo-β-galactosidase resistant, then we would expect the 2,4-dimethylgalactose to be present at a level of only 5.7% of the total galactose. We believe this is in good agreement with our data.

The position of the Galα1,3Galβ1,4GlcNAcβ1,6 branching structure of the I determinant along the poly(lactosamine) chain remains to be established. Indeed, this may be a form of microheterogeneity in the carbohydrate structure of laminin.

We do not have methylation data demonstrating the presence of the 2,3,4,6-tetra-*O*-methylmannose we would expect from the hybrid structures. We estimate that this species would exist at no more than 4% of the total 2,4-di-*O*-methylmannose. This would be almost a 10-fold lower level than 2,4-di-*O*-methylgalactose and well below our limits of detection.

On the basis of the results of acetolysis we believe that the short chains exist primarily linked Manα1,3Manβ1. This suggests that the blood group I structure would more probably be found on the Manα1,6Man branch. In addition, the exoglycosidase digestions suggest that the poly(lactosamine) chains contain at least two lactosaminyl units on one of the branches.

We have also demonstrated in laminin the presence of carbohydrate moieties that contain one long unbranched chain and a second, short chain with the sequence Galα1,3Galβ1,4GlcNAcβ1,2Manα1,3/6Man (1) or Galβ1,4GlcNAcβ1,2Manα1,3/6Man (2). These are novel structures (Table VI, structures II and IV), as are the biantennary structures containing the blood group I determinant on one branch and a second short chain terminated with sialic acid (Table VI, structure V).

Evidence for the presence of Galβ1,4GlcNAc units is also indicated by a previous study in our laboratory in which α-galactosidase-treated laminin was used as the substrate for a purified Ehrlich tumor cell α1,3-galactosyltransferase, which requires Galβ1,4GlcNAc as the substrate. It was shown that large amounts of [^{14}C]galactose were incorporated from [^{14}C]UDP-Gal (Elices et al., 1986).

The Con A reactive and Con A unreactive oligosaccharides were compared with regard to their reactivity with the GS I lectin. We found that 70% of the Con A reactive oligosaccharides bind to GS I–Sepharose, while only 50% of the Con A unreactive oligosaccharides interact with the lectin column (data not shown). It is possible that the addition of the α-D-Gal*p* residue is affected by the degree of branching, so that the more highly branched oligosaccharides might sterically hinder the efficient addition of α-D-Gal*p* residues by the galactosyltransferase. In this way tri- or tetraantennary structures would be less highly substituted with terminal α-D-Gal*p* units.

We have also presented evidence that the terminal sialic acid residues are linked NeuNAcα2,3Gal. These results include the precipitin reaction with the lectin from *M. amurensis* seeds, but not with the lectin from elderberry bark; sensitivity to New Castle disease virus neuraminidase, which is specific for NeuNAcα2,3 linkages; and the absence of 2,3,4-tri-*O*-methylgalactose upon methylation analysis. The presence in laminin of only α2,3-linked NeuNAc, rather than a mixture of α2,3- and α2,6-linked NeuNAc, is uncommon. This has been reported only for hCG (Endo et al., 1979), the proteoglycan from the Swarm rat chondrosarcoma (Lohmander et al., 1980), and human placental β-glucocerebrosidase (Takasaki et al., 1984).

It is unclear why endo H treatment was unable to release the hybrid oligosaccharides of laminin, which account for 8% of the Con A reactive oligosaccharides (<2% of the total oligosaccharides). It is likely that the low abundance of these structures on the laminin molecule or inefficient release from the peptide backbone precluded their detection. Further work is required to determine the structure of the complex arm as well as to more precisely determine the number of mannose residues on these species.

While this paper was in preparation Fujiwara and coworkers (Fujiwara et al., 1988) reported the distribution of oligosaccharides in the laminin domains. We have confirmed the presence of Galα1,3 linkages and the existence of poly(lactosaminyl) chains on the biantennary oligosaccharides.

Eckhardt and Goldstein (1983b) reported only 0.3–0.5 NeuNAc residue/3 Man residues in GP-130 from the Ehrlich ascites tumor cell. In contrast, our results demonstrate that laminin contains more terminal NeuNAc units than α-D-Gal groups. There is also evidence for terminal nonreducing β-D-Gal residues present on laminin. Additionally, methylation analysis of GP-130 reveals 3,4- and 3,6-di-*O*-methylmannose, but no 3,4,6-tri-*O*-methylmannose, clearly indicating that bi- and triantennary oligosaccharides do not exist in that structure. In contrast, methylation analysis of laminin reveals an abundance of 3,4,6-tri-*O*-methylmannose, as well as 3,4-di-*O*-methylmannose, indicating that laminin contains bi- and triantennary oligosaccharides, but no tetraantennary structures. Methylation of both GP-130 and laminin yielded 2,4-di-*O*-methylgalactose, indicating the presence of the blood group I structure. However, laminin displayed a lower abundance of this methylated derivative, suggesting that this structure occurs less frequently in laminin. Consistent with this is the sensitivity of the laminin oligosaccharides to endo-β-galactosidase, compared to insensitivity of intact GP-130 to endo-β-galactosidase.

We have been interested in the relationship of glycosylation patterns to transformation, activation, and malignancy in several murine systems: the Erhlich ascites tumor cell GP-130 (Eckhart & Goldstein 1983a,b), murine peritoneal macrophages (Maddox et al., 1982), and EHS laminin. This paper has described several unique oligosaccharide structures from the EHS tumor. It is important to continue this work by examining oligosaccharides from normal tissues to better understand the changes related to transformation in the murine model.

Other investigators have reported changes in glycosylation patterns upon transformation. Zhu and Laine (1985) reported that the longer poly(lactosamine) chains present on human fetal placental fibronectin reduced the interaction of fibronectin with gelatin. They also demonstrated that the enzymatic removal of the poly(lactosamine) chains increased the avidity of the fibronectin for gelatin. More recently, Dennis et al. (1987) and Pierce and Arango (1986) have reported that the GlcNAcβ1,6Manα1,6Man branch appears to be correlated with viral transformation in baby hamster kidney cells. Debray

et al. (1986) reported that for four human cell lines tumorigenicity was correlated with increased proportions of tri- and tetraantennary oligosaccharides and decreased biantennary structures. In addition, Yamamoto et al. (1984) reported that transformed thyroid glands produced thyroglobulin with poly(lactosamine)-containing oligosaccharide structures.

These studies suggest several features that might prove to be common to transformed cells. Tumor-specific structures would provide us with targets for monoclonal antibodies or lectins coupled to toxins and drugs. Structural features that may contribute to the metastatic capability of a cell, such as the poly(lactosamine) chains, might require that we target the regulation of specific metabolic pathways. It is first necessary to isolate and characterize laminin from nontransformed cells to compare features of the oligosaccharides. In this way we hope to further elucidate those structures important to the function of this molecule in vivo.

Added in Proof

Additional support for the presence of blood group i and I antigens in laminin is forthcoming from the fact that human antisera i(Den) and I(Step) (generously provided by B. Croucher and M. C. Crookston, Toronto General Hospital) both reacted strongly with laminin, as determined by ELISA.

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Environmental Modulation of M13 Coat Protein Tryptophan Fluorescence Dynamics†

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ABSTRACT: The effects of detergent [deoxycholate (DOC)] and phospholipid [dimyristoylphosphatidylcholine (DMPC)] environments on the rotational dynamics of the single tryptophan residue 26 of bacteriophage M13 coat protein have been investigated by using time-resolved single photon counting measurements of the fluorescence intensity and anisotropy decay. The total fluorescence decay of tryptophan-26 is complex but rather similar in DOC as compared to DMPC when analyzed in terms of a lifetime distribution (exponential series method). This similarity, in conjunction with the almost identical steady-state fluorescence spectra, indicates only minor differences between the tryptophan environments in DOC and DMPC. The reorientational dynamics of tryptophan-26 are dominated by slow rotation of the entire protein in both detergent and phospholipid environments. The resolved anisotropy decay in DOC can be approximated by a simple hydrodynamic model of protein/detergent micelle rotational diffusion, although the data indicate slightly greater complexity in the rotational motion. The tryptophan fluorescence anisotropy is not sensitive to protein conformational changes in DOC detected by nuclear magnetic resonance on the basis of pH independence in the range 7.5–9.1. In DMPC bilayers, restricted tryptophan motion with a correlation time of approximately 2 ns is observed together with a second very slow reorientational component. Resolution of the time constant for this slow rotation is obscured by the tryptophan fluorescence time window being too short to clearly locate its anisotropic limit. The possible contribution made by axial rotational diffusion of the protein to this slow rotational process is discussed. The fluorescence intensity and anisotropy decays are sensitive to the DMPC thermal phase transition, indicating that tryptophan-26 is in direct contact with phospholipid acyl chains.

The major coat protein of bacteriophage M13 (M13-CP)[1] and the homologous fd and fl coat proteins are comprised of 50 amino acid residues (M_r 5240) in a sequence that has a predominantly acidic N-terminal region, a basic C-terminal region, and a highly hydrophobic core (Asbeck et al., 1969; Nakashima & Konigsberg, 1974). The core region (residues 21–39) contains a single tryptophan residue at position 26. The protein is of interest in terms of bacteriophage assembly

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[1] Abbreviations: M13-CP, bacteriophage M13 gene 8 (coat) protein; DOC, sodium deoxycholate; DMPC, dimyristoylphosphatidylcholine; NMR, nuclear magnetic resonance; CD, circular dichroism; 16-DS, 16-doxylstearic acid.

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