Structures of the asparagine-linked oligosaccharides of guinea-pig Factor B of the alternative complement pathway

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This paper describes the structures of the asparagine-linked oligosaccharides of two forms of guinea-pig Factor B of the alternative complement pathway with different M_r values. Oligosaccharides were quantitatively liberated from both glycoproteins by hydrazinolysis, fractionated by paper electrophoresis and Bio-Gel P-4 column chromatography, and their structures determined by sequential exoglycosidase digestions in conjunction with methylation analysis. Both glycoproteins were shown to have the same biantennary complex-type oligosaccharides but it is suggested that they contain different numbers of oligosaccharide chains.

INTRODUCTION

In the alternative complement pathway, Factor B, which is a plasma glycoprotein, binds to C3b and is then cleaved into Ba and Bb fragments by Factor D, releasing the Ba fragment. The resulting complex (C3b–Bb) shows C3 convertase activity, which amplifies the formation of C3a and C3b from C3 [1]. Recently, two forms of Factor B with different M_r values have been isolated from guinea-pig plasma and characterized [2]. The M_r values of the two Factor B species, Factors B1 and B2, were estimated to be about 100 000 and 96 000 respectively by SDS/PAGE. Incubation of either form of Factor B with guinea-pig C3 and human Factor D in the presence of Mg^{2+} generated two cleavage fragments, Ba and Bb. Although both Bb fragments gave rise to molecules with the same M_r value of 62 000, the Ba fragments derived from Factors B1 and B2 exhibited M_r values of 38 000 and 34 000 respectively. Since both Ba fragments had an M_r of 30 000 upon digestion with endoglycosidase F, it was suggested that a difference in the asparagine-linked oligosaccharide moieties of the Ba fragments in guinea-pig plasma gave rise to the two forms of Factor B with different M_r values [2]. In order to arrive at a structural explanation of the difference in M_r , we investigated structures of their asparagine-linked oligosaccharide chains. The present paper indicates that, although the structures of the oligosaccharide chains of Factors B1 and B2 were identical, the numbers of chains were different.

MATERIALS AND METHODS

Materials, chemicals and enzymes

Factor B1 (M_r about 100 000) and Factor B2 (M_r about 96 000) were prepared from plasma of Hartley guinea pigs as previously described [2]. NaB^3H_4 (22.2 GBq/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Concanavalin A–Sepharose (approx. 10 mg of lectin/ml of gel sediment) was from Pharmacia (Uppsala, Sweden). Maltotetraose was from Nacalai Tesque (Kyoto, Japan). Radioactive oligosaccharides used in this study were obtained as described previously [3]. α -Mannosidase, β -galactosidase and β -N-acetylhexosaminidase were purified from jack-bean meal according to the methods of Li & Li [4]. Diplococcal β -galactosidase and diplococcal β -N-acetylglucos-

aminidase were purified according to the method of Glasgow *et al.* [5]. Sialidase purified from *Arthrobacter ureafaciens* was from Nacalai Tesque. Snail β -mannosidase purified from *Achatina fulica* was from Seikagaku Kogyo Co. (Tokyo, Japan). Digestions of the radioactive oligosaccharides (1×10^4 – 6×10^4 c.p.m.) with glycosidases were performed as reported previously [6].

Liberation of the asparagine-linked oligosaccharide chains of guinea-pig Factors B1 and B2

Factor B1 (5.6 mg) and Factor B2 (5.4 mg) were each suspended in 0.5 ml of anhydrous hydrazine and subjected to hydrazinolysis for 9 h followed by *N*-acetylation to liberate the asparagine-linked oligosaccharides quantitatively [7,8]. One-fifth portions of the oligosaccharides liberated from the glycoproteins were reduced with NaB^3H_4 (37 MBq) in 100 μ l of 0.05 M-NaOH at 30 °C for 4 h to obtain tritium-labelled oligosaccharides. Then 1 mg of $NaBH_4$ was added to each of the two preparations and the reaction was continued for an additional 2 h to complete the reduction. Radioactivities incorporated into the oligosaccharides were 2.7×10^6 c.p.m. and 2.1×10^6 c.p.m. for Factors B1 and B2 respectively. The counting efficiency of radioactivities was about 30 %. The remainder of the oligosaccharide fraction was reduced with 4 mg of NaB^3H_4 in 400 μ l of 0.05 M-NaOH at 30 °C for 4 h to obtain oligosaccharides for methylation analysis.

Determination of numbers of the asparagine-linked oligosaccharide chains of guinea-pig Factors B1 and B2

The numbers of asparagine-linked oligosaccharide chains per molecule of Factors B1 and B2 were determined by monitoring the release of oligosaccharides from 0.5 mg each of Factors B1 and B2 with time (5 h, 10 h and 15 h hydrazinolysis) by using the following modification of a published procedure [7–9]; *N*-acetylation was performed after addition of maltotetraose (10.3 nmol) to each reaction tube as an internal standard. After *N*-acetylation the oligosaccharide fraction was reduced with NaB^3H_4 (22.2 MBq) and the radioactive oligosaccharide mixture was subjected to paper chromatography in ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.) for 1 day. Radioactive oligosaccharides were eluted from the paper, evaporated to dryness and then desialylated by sialidase treatment. The

Abbreviations used: Sia, sialic acid; subscript OT is used to indicate NaB^3H_4 -reduced oligosaccharides; all sugars mentioned in this paper have the D-configuration.

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desialylated neutral oligosaccharide mixture was purified by paper electrophoresis at pH 5.4 and then applied to a Bio-Gel P-4 (extrafine grade) column to separate the radioactive asparagine-linked oligosaccharide fraction from the radioactive maltotetraol. On the basis of the radioactivities incorporated into the two fractions and of the M_r values of Factors B1 and B2 (100 000 and 96 000 respectively), the number of the asparagine-linked oligosaccharides released from 1 mol of the glycoprotein was calculated.

Analytical methods

Methylation analysis of oligosaccharides was performed with a Shimadzu GC-MS QP-1000 apparatus as reported previously [10]. Paper chromatography, high-voltage paper electrophoresis, Bio-Gel P-4 (extrafine grade) column chromatography, column chromatography on concanavalin A-Sepharose and other experimental procedures used in this study have been described previously [3,9,11,12].

RESULTS AND DISCUSSION

Separation by charge of oligosaccharides liberated from guinea-pig Factors B1 and B2

When both tritium-labelled oligosaccharide fractions obtained from Factors B1 and B2 were subjected to paper electrophoresis at pH 5.4, both samples gave one neutral (N) and two acidic (A1 and A2) fractions, as shown in Fig. 1. The percentage molar proportions of the radioactive oligosaccharides N, A1 and A2 were not significantly different for Factors B1 and B2 (10:32:58 for Factor B1 and 13:30:57 for Factor B2). Both acidic oligosaccharides were completely converted into neutral components after incubation with sialidase. On mild acid hydrolysis (0.01 M-HCl at 100 °C for 3 min), after which a part of the original acidic component still remained, fraction A1 gave an additional neutral component, whereas fraction A2 gave another acidic component with the same mobility as fraction A1, as well as a neutral component (results not shown). These results indicate that the acidic oligosaccharides A1 and A2 were monosialylated and disialylated oligosaccharides respectively.

Structures of oligosaccharides from Factors B1 and B2

In order to determine the anomeric configuration and sequence of each monosaccharide, the radioactive neutral oligosaccharide mixtures obtained by sialidase treatment of radioactive oligosaccharide fractions derived from Factors B1 and B2 were subjected to sequential exoglycosidase digestion followed by analysis of the products by chromatography on a Bio-Gel P-4 column.

Both radioactive neutral oligosaccharide mixtures gave a single peak that was eluted at a position corresponding to about 13

Fig. 1. Paper electrophoresis of oligosaccharides liberated from guinea-pig Factor B (B1) by hydrazinolysis

Arrows indicate the positions to which authentic lactitol (1) and 6'-sialyl-lactitol (2) migrated.

Fig. 2. Bio-Gel P-4 column chromatography of the radioactive desialylated oligosaccharide mixtures and exoglycosidase digests

The black arrows indicate the elution positions of glucose oligomers (numbers indicate the glucose units) added as internal standards. The white arrows indicate the elution positions of authentic oligosaccharides: I, $\text{Gal}\beta 1\text{-4GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{Gal}\beta 1\text{-4GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$; II, $\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$; III, $\text{Man}\alpha 1\text{-6}(\text{Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$. (a) and (b) show the radioactive oligosaccharide mixture obtained by sialidase treatment of oligosaccharides liberated from Factors B1 and B2 respectively; (c) shows the radioactive peak in (a) after incubation with jack-bean β -galactosidase; (d) shows the radioactive peak in (c) after digestion with jack-bean β -N-acetylhexosaminidase.

glucose units, the same position as authentic $\text{Gal}\beta 1\text{-4GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{Gal}\beta 1\text{-4GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$ (Figs. 2a and 2b). When incubated with jack-bean β -galactosidase, the radioactive neutral oligosaccharide from Factor B1 (Fig. 2a) released two galactose residues and yielded a smaller oligosaccharide that was eluted at the same position as authentic $\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$ (Fig. 2c). After incubation with jack-bean β -N-acetylhexosaminidase, the radioactive products, shown in Fig. 2(d) as a single peak that was eluted at the same position as authentic $\text{Man}\alpha 1\text{-6}(\text{Man}\alpha 1\text{-3})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}_{\text{OT}}$, demonstrated the release of two N-acetylglucosamine residues. By further sequential digestion with α -mannosidase, β -mannosidase and jack-bean β -N-acetylhexosaminidase (results not shown), the radioactive oligosaccharide in Fig. 2(d) was identified as a trimannosyl core structure of $\text{Man}\alpha 1\text{-(Man}\alpha 1\text{)-Man}\beta 1\text{-GlcNAc}\beta 1\text{-GlcNAc}_{\text{OT}}$. These results indicate that the peak in Fig. 2(d) was derived from $\text{Gal}\beta 1\text{-GlcNAc}\beta 1\text{-Man}\alpha 1\text{-(Gal}\beta 1\text{-GlcNAc}\beta 1\text{-Man}\alpha 1\text{)-Man}\beta 1\text{-GlcNAc}\beta 1\text{-GlcNAc}_{\text{OT}}$.

In a separate experiment, the radioactive oligosaccharide in Fig. 2(a) yielded the same products as those in Figs. 2(c) and 2(d) respectively upon sequential incubation with diplococcal β -galactosidase and diplococcal β -N-acetylglucosaminidase. It was also found that the radioactive neutral oligosaccharide mixture derived from Factor B1, as shown in Fig. 2(a), was bound to the concanavalin A-Sepharose column and eluted with buffer containing 0.1 M-methyl α -D-mannoside (results not shown). Since diplococcal β -galactosidase cleaves the $\text{Gal}\beta 1\text{-4GlcNAc}$ linkage but not $\text{Gal}\beta 1\text{-3GlcNAc}$ and $\text{Gal}\beta 1\text{-6GlcNAc}$ linkages [13],

Fig. 3. Proposed structures and proportions of the oligosaccharide chains of guinea-pig Factor B (B1 and B2) of the alternative complement pathway

and diplococcal β -*N*-acetylglucosaminidase hydrolyses the GlcNAc β 1-2Man linkage but not GlcNAc β 1-4Man and GlcNAc β 1-6Man linkages [14], and because binding to concanavalin A-Sepharose requires the presence of at least two unsubstituted α -mannosyl residues at positions C-3, C-4 and C-6 [12], it was concluded that the desialylated oligosaccharide derived from Factor B1 has a biantennary complex-type structure of Gal β 1-4GlcNAc β 1-2Man α 1-6(Gal β 1-4GlcNAc β 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc_{OT}. This conclusion was further supported by detection using methylation analysis of 3,4,6-tri-*O*-methylmannitol, 2,4-di-*O*-methylmannitol, 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol and 1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol as derivatives of substituted sugars of the desialylated oligosaccharide (results not shown).

The desialylated neutral oligosaccharide mixture from Factor B2 gave identical results with those obtained with that from Factor B1. Therefore structures of the desialylated oligosaccharides (shown in Figs. 2a and 2b respectively) derived from Factors B1 and B2 are the same.

Methylation analysis of oligosaccharide mixtures derived from Factors B1 and B2 revealed 2,3,4-tri-*O*-methylgalactitol in high abundance and 2,3,4,6-tetra-*O*-methylgalactitol in low abundance as galactose derivatives. In contrast, in the desialylated neutral oligosaccharide mixtures from Factors B1 and B2, large amounts of 2,3,4,6-tetra-*O*-methylgalactitol were detected whereas 2,3,4-tri-*O*-methylgalactitol was absent. Other derivatives were detected in almost the same proportions before and after sialidase treatment. Therefore it was concluded that sialic acid residues are linked to the C-6 position of terminal galactose residues of the oligosaccharide of Factors B1 and B2.

Comparison of the oligosaccharide moieties of Factors B1 and B2

The results described thus far indicate that the asparagine-linked oligosaccharide chains of guinea-pig Factors B1 and B2 of the alternative complement pathway have the same structure and are biantennary complex-type chains with zero, one and two sialic acid residues, as shown in Fig. 3.

It has been reported that B1-Ba and B2-Ba, the Ba fragments derived from Factors B1 and B2 by incubation with guinea-pig C3 and human Factor D in the presence of Mg²⁺, showed different mobilities on SDS/PAGE corresponding to *M_r* values of about 38000 and about 34000 respectively. In addition, digestions of both fragments with endoglycosidase F resulted in bands with the same *M_r* value of about 30000 [2]. Therefore it was suggested that the asparagine-linked oligosaccharide moieties of Factors B1 and B2 were different [2]. Although the present study indicates that the structures of the asparagine-linked oligosaccharide chains of Factors B1 and B2 are identical, it is demonstrated that the numbers of the asparagine-linked oligosaccharide chains are different.

The numbers of oligosaccharide chains per molecule of Factors B1 and B2 were determined to be about four and three

respectively, on the basis of the radioactivities incorporated into the asparagine-linked oligosaccharide as well as maltotetraose added as an internal standard and on the *M_r* values of Factors B1 and B2 (100000 and 96000 respectively) as described in the Materials and methods section. This suggests that Factor B1 contains one additional oligosaccharide chain as compared with Factor B2. Sialic acid residues on cell membranes have been demonstrated to regulate complement activation [15,16]. Therefore it is conceivable that the additional glycosylation that increases the sialic acid content on Factor B1 molecules might diminish complement reactivity. However, no difference in biological function measured by haemolytic assay was observed between Factors B1 and B2. Since no difference in antigenicity was noted between Factors B1 and B2, and since the *N*-terminal amino acid sequences of the two glycoproteins and their Bb fragments were identical [2], it is suggested that the potential *N*-glycosylation site not glycosylated in Factor B2 was glycosylated in Factor B1, which would explain the difference in *M_r*. For confirmation of this idea, the determination of the complete amino acid sequences of these two glycoproteins is essential.

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