Structural Study of the Sugar Chains of CD36 Purified from Bovine Mammary Epithelial Cells: Occurrence of Novel Hybrid-Type Sugar Chains Containing the Neu5Acα2→6GalNAcβ1→4GlcNAc and the Manα1→2Manα1→3Manα1→6Man Groups†

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ABSTRACT: CD36 is a glycoprotein included in the bovine milk fat globule membrane derived from mammary secretory epithelial cells during lactation. Asparagine-linked sugar chains were quantitatively released from CD36 as oligosaccharides by hydrazinolysis. These sugar chains were converted to radioactive oligosaccharides by reduction with NaB^3H_4 and separated into neutral and acidic fractions by paper electrophoresis. Most of the acidic oligosaccharides were converted to neutral ones by sialidase digestion, indicating that they are sialyl derivatives. The neutral and sialidase-treated acidic oligosaccharides were fractionated by Bio-Gel P-4 column chromatography in combination with serial chromatography on immobilized lectin columns including a *Wistaria floribunda* agglutinin (WFA)-agarose column. WFA is known to bind oligosaccharides terminating with either an α- or β-*N*-acetylgalactosamine residue. Structural studies of oligosaccharides in each fraction by sequential exoglycosidase digestion as well as methylation analysis revealed that CD36 contains high mannose-type, hybrid-type, and bi-, tri-, and tetraantennary complex-type sugar chains. A portion of the hybrid-type and the complex-type sugar chains which bound to a WFA-agarose column (28% of all oligosaccharides) contained the GalNAcβ1→4GlcNAc group(s) instead of the Galβ1→4GlcNAc group(s) in their outer chain moieties. Like oligosaccharides found in human luteinizing hormone [Weisshaar, G., Hiyama, J., Renwick, A. G., & Nimtz, M. (1991) *Eur. J. Biochem. 195*, 257–268], some of the GalNAcβ1→4GlcNAc groups found in the CD36 oligosaccharides were sialylated as the Neu5Acα2→6GalNAc group. Furthermore, most of the hybrid-type sugar chains of CD36 with the Gal/GalNAcβ1→4GlcNAcβ1→2 outer chain on their Manα1→3 arm contained an unusual Manα1→2Manα1→3 group on their Manα1→6 arm.

CD36 is a glycoprotein present at the cell surface of platelets, capillary endothelial cells, erythroblasts, monocytes and mammary epithelial cells (Knowles et al., 1984; Edelman et al., 1986; Tandon et al., 1989a; Greenwalt et al., 1990a). CD36 is thought to be involved in the initial phase of collagen fibril–platelet interaction (Tandon et al., 1989b; Tandon et al., 1991) and has been proposed as the membrane receptor for thrombospondin (TSP)[1] (Asch et al., 1987; McGregor et al., 1989), thus mediating the TSP-dependent adhesion of monocytes to macrophages and platelets (Silverstein et al., 1989). Furthermore, CD36 has been shown to mediate binding of erythrocytes infected with *Plasmodium falciparum* to capillary endothelial cells during the course of the malarial infection (Ockenhouse et al., 1989).

During lactation, milk triacylglycerols are secreted from mammary epithelial cells as droplets covered by a layer of the apical membrane of the cells [reviewed by Mather and Keenan (1983)]. This membrane layer is referred to as the milk fat globule membrane (MFGM) and contains a limited number of proteins [reviewed by Mather (1987)]. One of them is the "PAS IV" glycoprotein, which has been shown to be identical to CD36 present in platelets and endothelial cells (Greenwalt et al., 1990a). Platelet CD36 has been shown to be associated with the tyrosine kinases, Fyn, Lyn, and Yes, and CD36–collagen interaction is supposed to activate CD36-mediated signaling pathways by tyrosine phosphorylation, causing the rigid attachment of platelet to collagen matrix (Huang et al., 1991). However, the function of mammary epithelial cell CD36 is unknown.

Since the molecular size of CD36 is quite different among tissues, probably due to differences in glycosylation (Greenwalt et al., 1990a,b), an extensive structural study of the sugar chains of bovine MFGM CD36 has been performed. The data indicated that the glycoprotein contains unusual asparagine-linked sugar chains including novel hybrid-type sugar chains, the formation of which cannot be explained by any currently known biosynthetic pathway.

EXPERIMENTAL PROCEDURES

Purification of Bovine Milk Fat Globule Membrane (MFGM) CD36. Bovine MFGM CD36 was purified as described by Greenwalt et al. (1990a). In brief, bovine MFGM was extracted with 1% Triton X-114 (5 mg of protein/mL) in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS). After being stirred at 4 °C for 30 min, the mixture

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[1] Abbreviations: MFGM, milk fat globule membrane; TSP, thrombospondin; Con A, concanavalin A; AAL, *Aleuria aurantia* lectin; WFA, *Wistaria floribunda* agglutinin; DSA, *Datura stramonium* agglutinin; RCA, *Ricinus communis* agglutinin; SNA, *Sambucus nigra* agglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A subscript OT is used to indicate an NaB^3H_4-reduced oligosaccharide. All sugars mentioned in this paper have the D-configuration except for fucose which has the L-configuration.

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was centrifuged at 100000*g* for 2 h. The supernatant was subjected to phase partitioning by warming to 30 °C and centrifugation at 1000*g* for 10 min. The detergent phase was diluted 10-fold with TBS, cooled to 4 °C, and repartitioned. The resultant detergent phase was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 (TT). The sample was then chromatographed sequentially on carboxymethyl-Sepharose columns at pH values of 8, 7, and 6, respectively. CD36 was eluted from each column with TT containing 0.1 M NaCl.

Liberation of Asn-Linked Sugar Chains from CD36. CD36 (10 mg), which was dried thoroughly over P_2O_5 in vacuo, was subjected to hydrazinolysis for 15 h as described previously (Takasaki et al., 1982). After *N*-acetylation, two-thirds of the liberated oligosaccharide mixture from CD36 was reduced with NaB^3H_4 to obtain tritium-labeled oligosaccharides for structural analysis, and the remainder was reduced with NaB^2H_4 to obtain deuterium-labeled oligosaccharides for methylation analysis. To facilitate the detection of the deuterium-labeled oligosaccharides, one-tenth of the tritium-labeled oligosaccharides was added.

In order to determine the number of asparagine-linked sugar chains included in one molecule of CD36, a time course study of the liberation of oligosaccharides by hydrazinolysis was performed using 100 μg of CD36 for each incubation time. Xylose (25 nmol) as an internal standard was added to the reaction mixture prior to reduction with NaB^3H_4. The radioactive oligosaccharides and [^{3}H]xylitol were separated by paper chromatography using 1-butanol–ethanol–water (4:1:1, v/v) as a solvent.

Sugar Composition and Reducing Terminal Sugar Analyses. Since some of the oligosaccharides released from CD36 by hydrazinolysis were totally resistant to diplococcal β-galactosidase, streptococcal 6646 K β-galactosidase, or diplococcal β-*N*-acetylhexosaminidase digestion, analysis of the sugar composition as well as the reducing terminal sugar was performed on the oligosaccharides according to the method of Takeuchi et al. (1987). In brief, half of the oligosaccharide fraction which was prepared by paper chromatography of the oligosaccharides released from CD36 (1 mg) by hydrazinolysis (100 °C, 20 h) using 1-butanol–ethanol–water (4:1:1, v/v) was reduced with NaB^3H_4 and then hydrolyzed with 4 N HCl at 100 °C for 4 h, and the remaining half was hydrolyzed with 4 N HCl at 100 °C for 4 h and then reduced with NaB^3H_4, and they were re-*N*-acetylated. The radioactive sugars were mixed with standard monosaccharide alcohols and injected into the HPLC system with use of a Shodex SUGAR SP-1010 column. The elution positions of each monosaccharide alcohol were detected by the refracto-index monitor.

Analytical Methods. The radioactive oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine–acetate buffer (3:1:387, pyridine:acetic acid:water), pH 5.4, at 70 V/cm for 90 min. Fractionation of the radioactive oligosaccharides by Bio-Gel P-4 column chromatography was performed as reported by Yamashita et al. (1982). Methylation analysis and periodate oxidation of oligosaccharides were conducted as described in previous papers [Furukawa et al. (1989) and Yamashita et al. (1981), respectively]. Identification of sialic acid released from CD36 by *Arthrobacter ureafaciens* sialidase was performed by paper chromatography using 1-butanol–acetic acid–water (12:3:5) after NaB^3H_4 reduction (Mizuochi et al., 1981).

Affinity Chromatography of Oligosaccharides on Immobilized Lectin Columns. Lectin column chromatography using immobilized *Aleuria aurantia* lectin (AAL), *Wistaria floribunda* agglutinin (WFA), concanavalin A (Con A), *Datura stramonium* agglutinin (DSA), *Ricinus communis*-I agglutinin (RCA-I), and *Sambucus nigra* agglutinin (SNA) was performed as described previously (Yamashita et al., 1985, 1987; Torres et al., 1988; Ogata et al., 1975; Harada et al., 1987; Shibuya et al., 1987a,b). In brief, samples on an AAL–Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, followed by the same buffer containing 1 mM L-fucose. Samples on a WFA–agarose column were eluted with phosphate-buffered saline, pH 7.4, followed by the same buffer containing 100 mM *N*-acetylgalactosamine. Samples on a Con A–Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, followed by the same buffer containing 5 mM α-methyl D-glucopyranoside and then with 100 mM-α-methyl D-mannopyranoside. Samples on a DSA–Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, followed by the same buffer containing 1% *N*-acetylglucosamine oligomers. Samples on an RCA-I–agarose column were eluted with phosphate-buffered saline, pH 7.4, followed by the same buffer containing 10 mM lactose. Samples on an SNA–agarose column were eluted with phosphate-buffered saline, pH 7.4, followed by the same buffer containing 100 mM lactose.

Chemcials, Enzymes, and Lectins. WFA–agarose and SNA–agarose were obtained from E. Y. Labs, San Mateo, CA. Con A–Sepharose and RCA-I–agarose were purchased from Pharmacia Fine Chemical Co., Tokyo and Honen Oil Co., Tokyo, respectively. DSA–Sepharose was kindly supplied from Wako Pure Chemical Co., Tokyo, and AAL–Sepharose from Nichirei Co., Tokyo. NaB^3H_4 (600 mCi/mmol) was purchased from New England Nuclear, Boston, MA, and NaB^2H_4, sialidase from *A. ureafaciens*, chlorotriphenylmethane, and β-methyl D-*N*-acetylgalactopyranoside were obtained from Nacalai Tesque Co., Kyoto. 6-*O*-Triphenylmethane(β-methyl D-*N*-acetylgalactosamine) was chemically synthesized according to the method of Bhattacharjee et al. (1981) with use of chlorotriphenylmethane and β-methyl D-*N*-acetylgalactosamine and used as a standard for 3,4-di-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified from culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow et al. (1977). Jack bean β-*N*-acetylhexosaminidase and α-mannosidase were purified from jack bean meal (Sigma Chemical Co., St. Louis, MO) by the method of Li and Li (1972). *D. pneumoniae endo*-β-*N*-acetylglucosaminidase D, *Streptomyces griseus endo*-β-*N*-acetylglucosaminidase H, snail β-mannosidase, and GM_2-ganglioside were kindly supplied from Seikagaku Kogyo Co., Tokyo. *Aspergillus saitoi* α-mannosidase I was purified according to the method of Kobata and Amano (1987).

Exoglycosidase Digestion. Unless otherwise mentioned, oligosaccharides (1–5 × 10^4 cpm) were incubated with one of the following mixtures at 37 °C for 18 h except that digestion with *endo*-β-*N*-acetylglucosaminidase D or *endo*-β-*N*-acetylglucosaminidase H was performed for 40 h: (1) *A. ureafaciens* sialidase (50 milliunits) in 50 μL of 0.5 M acetate buffer, pH 5.0; (2) diplococcal β-galactosidase (2 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 6.0; (3) diplococcal β-*N*-acetylhexosaminidase (4 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 6.0; (4) *Streptococcus* 6646 K β-galactosidase (5 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 5.5; (5) jack bean β-*N*-acetylhexosaminidase (0.5 unit) in 50 μL of 0.3 M citrate phosphate buffer, pH 4.0, containing 100 μg of γ-galactonolactone; (6) *A. saitoi*

α-mannosidase I (0.15 μg) in 30 μL of 0.5 M acetate buffer, pH 5.0; (7) jack bean α-mannosidase (0.8 unit) in 50 μL of 0.5 M acetate buffer, pH 4.5, containing 1 mM $ZnCl_2$; (8) snail β-mannosidase (10 milliunits) in 50 μL of 0.05 M sodium citrate buffer, pH 4.0; (9) bovine epididymis α-L-fucosidase (35 milliunits) in 50 μL of 0.2 M sodium citrate buffer, pH 6.0; (10) *D. pneumoniae endo-β-N*-acetylglucosaminidase D (100 milliunits) in 30 μL of 0.2 M citrate phosphate buffer, pH 6.0; (11) *S. griseus endo-β-N*-acetylglucosaminidase H (10 milliunits for oligosaccharides in fraction AAL^- and 100 milliunits for oligosaccharides in fraction AAL^+, respectively) in 30 μL of 0.15 M citrate phosphate buffer, pH 5.0. One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Digestions were terminated by heating the reaction mixture in a boiling water bath for 3 min, and the product was desalted and analyzed by Bio-Gel P-4 column chromatography.

Oligosaccharides. Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac$_2$·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$ and Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6 or 3(Galβ1→4GlcNAcβ1→2Manα1→3 or 6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) were prepared from human transferrin (Spik et al., 1975) by hydrazinolysis, and their desialylated oligosaccharide (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) was prepared by digestion with *A. ureafaciens* sialidase. Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was prepared from human platelet thrombospondin (Furukawa et al., 1989). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_3$·GlcNAc·GlcNAc$_{OT}$) and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4 (Fucα1→6)GlcNAc$_{OT}$ (Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$ and Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, respectively, by digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase. Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man·GlcNAc·GlcNAc$_{OT}$) and Manβ1→GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man·GlcNAc·Fuc·GlcNAc$_{OT}$) were prepared from Man$_3$·GlcNAc·GlcNAc$_{OT}$ and Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, respectively, by digestion with jack bean α-mannosidase. Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_5$·GlcNAc·GlcNAc$_{OT}$) was obtained from bovine pancreatic ribonuclease B (Liang et al., 1980).

RESULTS

Number and Sugar Composition of Asn-Linked Sugar Chains. The approximate number of asparagine-linked sugar chains that bound to 1 mol of CD36 was calculated to be 6 mol (Figure 1), on the basis of the radioactivities incorporated into xylitol and the oligosaccharides liberated by hydrazinolysis and the molecular weight of CD36 as 78 000 as described under Experimental Procedures. This value is consistent with that estimated from the decrease in the molecular weight of CD36 on SDS–PAGE after endoglycosidase treatment (Greenwalt et al., 1991).

The elution pattern of the radioactive monosaccharides obtained after the hydrolysis of the CD36 sugar chains and that of the reducing terminal sugar are shown in Figure 2A, panels A and B, respectively. These results indicated that the released oligosaccharide fraction contains *N*-acetylgalactosamine as a constitutive monosaccharide besides galactose, mannose, *N*-acetylglucosamine, and fucose, and the reducing terminal sugar is exclusively *N*-acetylglucosamine. Therefore, some of the asparagine-linked sugar chains should contain an *N*-acetylgalactosamine residue.

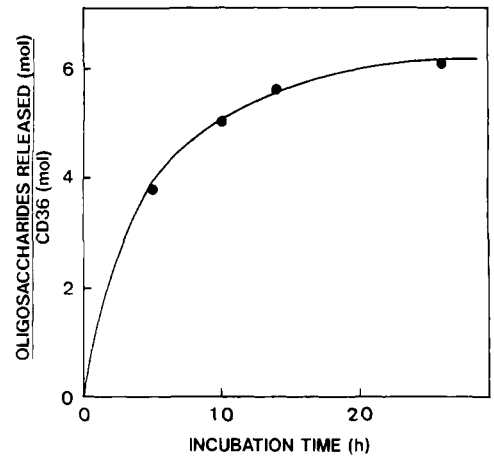


FIGURE 1: Time course of the release of asparagine-linked sugar chains from CD36 by hydrazinolysis. The amount of the sugar chains released from CD36 is calculated by determining the radioactivities incorporated into the oligosaccharide fraction and xylose, and the molecular weight of CD36, as described under Experimental Procedures.

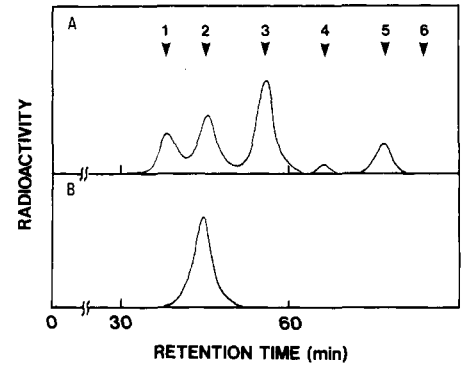


FIGURE 2: Analyses of carbohydrate composition (A) and reducing terminal sugar (B) of the sugar chains obtained from CD36. The oligosaccharide mixture obtained from CD36 by hydrazinolysis (100 °C, 20 h) was reduced with NaB^3H_4 before and after hydrolysis with 4 N HCl at 100 °C for 4 h. After re-*N*-acetylation, they were mixed with standard sugar alcohols and injected into the HPLC system with use of a Shodex SUGAR SP-1010 column. Panels A and B show the elution patterns of labeled alditols and *N*-acetylaminoalditols obtained from the oligosaccharide mixture labeled after and before the hydrolysis, respectively. The numbers at the top of the figure indicate the elution positions of standard sugar alcohols: (1) *N*-acetylgalactosaminitol; (2) *N*-acetylglucosaminitol; (3) mannitol; (4) fucitol; (5) galactitol; (6) glucitol.

Fractionation of Oligosaccharides by Paper Electrophoresis. The radioactive oligosaccharides released from CD36 by hydrazinolysis were subjected to paper electrophoresis and separated into neutral (N) and acidic (A) fractions (Figure 3a). The percent molar ratios of fractions N and A calculated from their radioactivities were 61% and 39%, respectively. When fraction A was digested exhaustively with *A. ureafaciens* sialidase, 95% of the acidic oligosaccharides was converted to neutral ones as shown in Figure 3b. Therefore, most of the acidic nature of the oligosaccharides could be ascribed to their sialic acid residues. Paper chromatographic analysis of the types of sialic acid residues, released from CD36 by sialidase

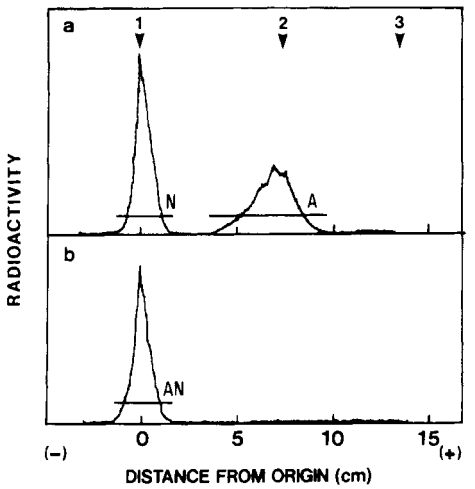


FIGURE 3: Paper electrophoresis of oligosaccharides released from CD36 (a) and of sialidase digests of the acidic oligosaccharides (indicated by a bar A) (b). The arrow heads at the top of the figure indicate the positions to which authentic oligosaccharides migrated: (1) lactitol; (2) Neu5Ac·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$; (3) $Neu5Ac_2$·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$.

Table I: Percent Molar Ratio of Oligosaccharides Fractionated by Serial Lectin Column Chromatography

	molar ratio (%)			
	N		AN	
fractions	AAL^-	AAL^+	AAL^-	AAL^+
I. WFA^-·$Con\ A^-$	1.0	1.6	2.3	3.9
II. WFA^-·$Con\ A^r$	1.3	2.5	2.9	4.9
III. WFA^-·$Con\ A^+$	2.1	1.8	4.5	7.2
IV. WFA^-·$Con\ A^{++}$	34.3	0.2	1.2	tr[a]
V. WFA^+·DSA^-·RCA^-	2.9	5.8	1.0	2.1
VI. WFA^+·DSA^-·RCA^r	1.8	4.3	1.5	5.7
VII. WFA^+·DSA^+	0.5	1.1	0.3	1.3

[a] Trace, less than 1%.

digestion followed by reduction with NaB^3H_4, revealed that all of them are *N*-acetylneuraminic acid (data not shown).

Fractionation of Oligosaccharides in Fractions N and AN by Serial Lectin Column Chromatography. Since sugar composition analysis of the oligosaccharide fraction obtained from CD36 by hydrazinolysis showed the presence of *N*-acetylgalactosamine in addition to mannose, galactose, fucose, and *N*-acetylglucosamine (Figure 2A), it was assumed that some of the asparagine-linked sugar chains contain this sugar residue as a penultimate sugar. Therefore, oligosaccharides in fractions N and AN were initially subjected to WFA–agarose column chromatography in order to separate oligosaccharides with and without an *N*-acetylgalactosamine residue at their nonreducing termini. The fraction that passed through the column was designated as WFA^- and that bound to the column and eluted with 100 mM *N*-acetylgalactosamine as WFA^+. As shown in Table I, approximately 28% of the total sugar chains bound to a WFA–agarose column. Oligosaccharides in the fraction WFA^- were applied to a Con A–Sepharose column. The pass-through fraction (WFA^-·$Con\ A^-$), the fraction that was retarded in the column (WFA^-·$Con\ A^r$), the bound fraction eluted with 5 mM α-methyl D-glucopyranoside (WFA^-·$Con\ A^+$), and the bound fraction eluted with 100 mM α-methyl D-mannopyranoside (WFA^-·$Con\ A^{++}$) were obtained. Oligosaccharides in the WFA^+ fraction were applied to a DSA–Sepharose column, and the pass-through fraction (WFA^+·DSA^-), and the bound fraction eluted with 1% *N*-acetylglucosamine oligomers (WFA^+·DSA^+) were obtained. Oligosaccharides in the fraction WFA^+·DSA^- were further separated by RCA-I–agarose column chromatography. The pass-through fraction (WFA^+·DSA^-·RCA-I^-) and the fraction that was retarded in the column (WFA^+·DSA^-·RCA-I^r) were obtained. The seven fractions thus obtained were named fractions I–VII as listed in Table I. Finally, oligosaccharides in each of the seven fractions were applied to an AAL–Sepharose column. The pass-through fraction (AAL^-) and the fraction bound to the column and eluted with 1 mM α-L-fucose (AAL^+) were obtained. The molar ratios of oligosaccharides in each fraction separated by the serial lectin column chromatography were calculated on the basis of the radioactivities of the fractions and are summarized in Table I.

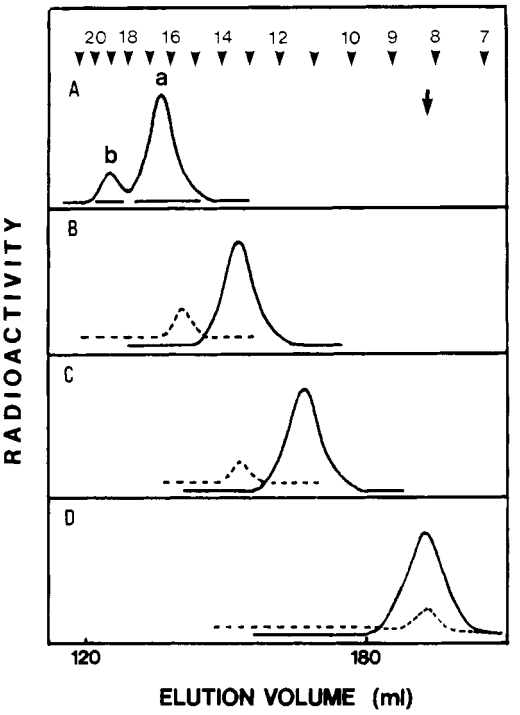


FIGURE 4: Sequential exoglycosidase digestion of fraction AN-I. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction AN-I; (B) solid and dotted lines indicate oligosaccharides a and b in panel A digested with diplococcal β-galactosidase, respectively; (C) solid and dotted lines indicate the oligosaccharides shown by solid and dotted lines in panel B digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (D) solid and dotted lines indicate the oligosaccharides shown by solid and dotted lines in panel C digested with jack bean β-*N*-acetylhexosaminidase, respectively. Arrowheads at the top of the figure indicate the elution positions of glucose oligomers used as internal standards, and the numbers indicate the glucose units. A black arrow indicates the elution position of authentic oligosaccharide Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$.

Structures of Oligosaccharides in Fraction I. Oligosaccharides in fraction AAL^+ of fraction I from AN (named AN-I), which contained 3.9% of the total oligosaccharides, were separated into two components a and b with effective sizes of 16.5 and 19.0 glucose units by Bio-Gel P-4 column chromatography (Figure 4A). When radioactive component a was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (solid line peak in Figure 4B) and one *N*-acetylglucosamine residue (solid line peak in Figure 4C) were released. The solid line peak in Figure 4C was further digested with jack bean β-*N*-acetylhexosaminidase, and the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, releasing two *N*-acetylglucosamine residues (solid line peak in Figure 4D).

When radioactive component b was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, four galactose residues (dotted line peak in Figure 4B) and one *N*-acetylglucosamine residue (dotted line peak in Figure 4C) were released. The dotted line peak in Figure 4C was further digested with jack bean β-*N*-acetylhexosaminidase, and the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, releasing three *N*-acetylglucosamine residues (dotted line peak in Figure 4D). That the solid and dotted line products in Figure 4D have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by sequential digestion with jack bean α-mannosidase, snail β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and bovine epididymis α-L-fucosidase, and methylation analysis (data not shown).

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL^+ of fraction N-I gave the same series of elution profiles of oligosaccharides described above. The elution profiles of oligosaccharides in fraction AAL^- of fractions AN-I and N-I and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of oligosaccharides in fraction AAL^+ of fraction AN-I, except that the elution position of each peak was smaller than the respective peak from AAL^+ fraction by one glucose unit, reflecting the absence of the fucose residue linked to the proximal *N*-acetylglucosamine residue.

On the basis of the specificity of diplococcal β-*N*-acetylhexosaminidase which cleaves the GlcNAcβ1→2Man linkage of the GlcNAcβ1→4(GlcNAcβ1→2)Man group but not that of the GlcNAcβ1→6(GlcNAcβ1→2)Man group, component a was considered to be a triantennary complex-type sugar chain having the GlcNAcβ1→6(GlcNAcβ1→2)Man group and component b a tetraantennary complex-type sugar chain as shown in Table IV.

Structures of Oligosaccharides in Fraction II. Oligosaccharide in fraction AAL^+ of fraction AN-II, which amounted to 4.9% of the total oligosaccharides, was eluted from a Bio-Gel P-4 column as a single peak (Figure 5A). When the peak in Figure 5A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue (Figure 5B) and one *N*-acetylglucosamine residue (Figure 5C) were released. When the peak in Figure 5C with an effective size of 9.8 glucose units was digested with *A. saitoi* α-mannosidase I, which cleaves only the Manα1→2Man linkage, the product was eluted at the position of 8.8 glucose units (Figure 5D), indicating that one mannose residue was removed. After digestion of the peak in Figure 5D with jack bean α-mannosidase, three mannose residues were released, and the product was eluted at the same position as authentic Man·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 5E). That the product in Figure 5E has the structure Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by sequential digestion with snail β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and bovine epididymis α-L-fucosidase and methylation analysis (data not shown).

Sequential exoglycosidase digestion of oligosaccharide in fraction AAL^+ of fraction N-II gave exactly the same series of results as described earlier for the component in Figure 5A. The elution profiles of oligosaccharides in fraction AAL^- of fractions AN-II and N-II and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL^+ of fraction AN-II, except that the elution position of each peak was smaller than the corresponding peak in the AAL^+ fraction by one glucose unit (data not shown).

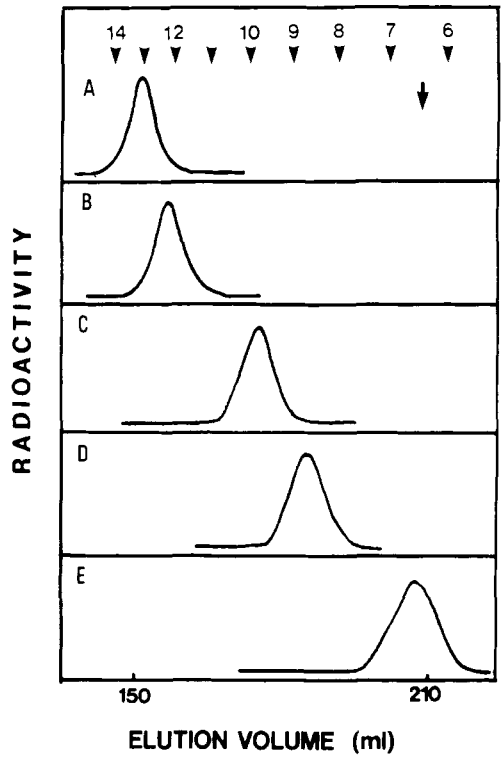


FIGURE 5: Sequential exoglycosidase digestion of fraction AN-II. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction AN-II; (B) oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) oligosaccharide in panel B digested with diplococcal β-*N*-acetylhexosaminidase; (D) oligosaccharide in panel C digested with *A. saitoi* α-mannosidase I; (E) oligosaccharide in panel D digested with jack bean α-mannosidase. Arrowheads at the top of the figure are the same as in Figure 4. A black arrow indicates the elution position of authentic oligosaccharide Man·GlcNAc·Fuc·$GlcNAc_{OT}$.

Comparison of methylation data of oligosaccharides in this fraction before and after digestion with *A. saitoi* α-mannosidase I showed that the amount of 3,4,6-tri-*O*-methylmannitol is reduced to a half of that after the enzyme digestion, but an equivalent amount of 2,3,4,6-tetra-*O*-methylmannitol was detected before and after the enzyme digestion (Table II), indicating that a mannose residue is linked at the C-2 position of another mannose residue. In order to determine to which of the arms of the trimannosyl core the Galβ1→4GlcNAc group is linked, oligosaccharides in fractions AAL^- and AAL^+ of fraction AN-II before and after digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase were treated with diplococcal *endo*-β-*N*-acetylglucosaminidase D (endo D). Both oligosaccharides were resistant to endo D digestion, while the degalactosylated and de-*N*-acetylglucosaminylated oligosaccharides were completely hydrolyzed by endo D and liberated radioactive *N*-acetylglucosaminitol and fucosyl *N*-acetylglucosaminitol, respectively (data not shown). Since endo D requires the general structure of R→Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc (in which R represents either hydrogen or sugars) for its substrate (Tai et al., 1975), the results indicated that the Galβ1→4 GlcNAc group should be linked to the Manα1→3 arm. When oligosaccharides in fractions AAL^- and AAL^+ of fraction AN-II were digested with *S. griseus endo*-β-*N*-acetylglucosaminidase H (endo H), they were completely digested with the enzyme and liberated radioactive *N*-acetylglucosaminitol and fucosyl

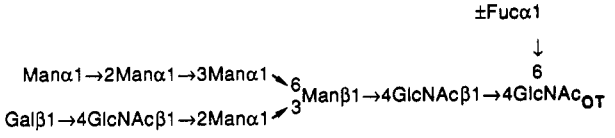
Table II: Methylation Analysis of Each Lectin Fraction Obtained from a Mixture of Fractions N and AN

partially *O*-methylated sugars	fractions, molar ratio[a]				
	WFA⁻·Con A⁻(I)	WFA⁻·Con A^r(II)	[WFA⁻·Con A^r(II)][b]	WFA⁻·Con A⁺(III)	WFA⁻·Con A⁺⁺(IV)
fucitol					
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.6	0.6	0.5	0.5	–
galactitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	3.1	0.8	0.9	1.8	0.1
mannitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	–	0.7	0.8	–	2.4
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	0.8	1.7	0.9	1.8	1.9
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	–	0.7	0.8	–	–
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.1	–	–	–	–
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.9	–	–	–	–
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	1.0	1.0	1.7
2-*N*-methylacetamido-2-deoxyglucitol					
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	3.9	1.6	1.7	2.5	1.0
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.3	0.4	0.3	0.4	0.8
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.6	0.5	0.5	0.4	–

[a] Numbers in the table were calculated by taking the value of 2,4-di-*O*-methylmannitol as 1.0, except for those in WFA⁻·Con A⁺⁺, which were calculated by taking the value of 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol as 1.0. [b] After *A. saitoi* α-mannosidase I digestion. [c] Not detectable.

N-acetylglucosaminitol (data not shown). Since endo H requires the general structure of $R_1 \rightarrow 6(R_2 \rightarrow$ Man$\alpha 1 \rightarrow 3)$Man$\alpha 1 \rightarrow 6(R_3 \rightarrow$ Man$\alpha 1 \rightarrow 3)$Man$\beta 1 \rightarrow 4$GlcNAc$\beta 1 \rightarrow 4$-($\pm$Fuc$\alpha 1 \rightarrow 6$)GlcNAc (in which R_1, R_2, and R_3 represent either hydrogen or sugars) for its substrate (Tai et al., 1977), the results indicated that the Man$\alpha 1 \rightarrow 2$Man$\alpha 1 \rightarrow 3$Man group should be linked to the Man$\alpha 1 \rightarrow 6$ arm. Digestion with endo D and endo H of oligosaccharides in fractions AAL⁻ and AAL⁺ of fraction N-II gave the same results as those described earlier for oligosaccharides in fractions AAL⁻ and AAL⁺ of fraction AN-II (data not shown).

These results indicated that the oligosaccharides in the fraction II are hybrid-type sugar chains having the following structures.



A hybrid-type oligosaccharide with the Man$\alpha 1 \rightarrow 6$(Man-$\alpha 1 \rightarrow 3$)Man$\alpha 1 \rightarrow 6$Man group which was detected in fraction IV binds to a Con A–Sepharose column and is eluted with 100 mM α-methyl D-mannopyranoside (Asada et al., 1991). However, the above oligosaccharides were only retarded in the column despite the presence of three mannose residues which theoretically interact with Con A. This is probably because the tertiary structures of the oligosaccharides are not favorable for binding to Con A.

Structures of Oligosaccharides in Fraction III. Oligosaccharides in fraction AAL⁺ of fraction AN-III, which contained 7.2% of the total oligosaccharides, was eluted from a Bio-Gel P-4 column as a single peak whose elution position is 14.2 glucose units (Figure 6A). When the peak in Figure 6A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (Figure 6B) and two *N*-acetylglucosamine residues were released and the product was eluted at the same position as authentic Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (Figure 6C). That the product has the structure Man$\alpha 1 \rightarrow 6$(Man$\alpha 1 \rightarrow 3$)-Man$\beta 1 \rightarrow 4$GlcNAc$\beta 1 \rightarrow 4$(Fuc$\alpha 1 \rightarrow 6$)GlcNAc$_{OT}$ was confirmed by methods already described. Structural study of the oligosaccharides in fraction AAL⁺ of fraction N-III by sequential exoglycosidase digestion gave the same series of the results as described for the oligosaccharide in Figure 6A

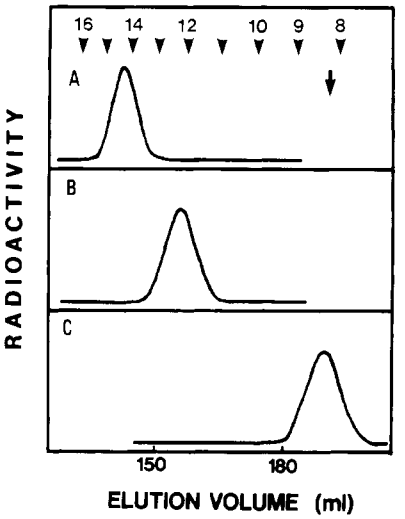


FIGURE 6: Sequential exoglycosidase digestion of fraction AN-III. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) AAL⁺ fraction of fraction AN-III; (B) oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) oligosaccharide in panel B digested with diplococcal β-*N*-acetylhexosaminidase. The arrowheads at the top of the figures and the black arrow are the same as in Figure 4.

(data not shown). The elution profiles of oligosaccharide in fraction AAL⁻ of fractions AN-III and N-III and its sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL⁺ of fraction AN-III, except that the elution position of each peak was smaller than the corresponding peak in the AAL⁺ fraction by one glucose unit (data not shown).

These results together with the methylation data indicated that oligosaccharides in fraction III are biantennary complex-type sugar chains with and without a fucose residue attached to the proximal *N*-acetylglucosamine residue as shown in Table IV.

Structures of Oligosaccharides in Fraction IV. This fraction contains 35.7% of the total oligosaccharides. When fraction AAL⁻ of fraction N-IV was subjected to Bio-Gel P-4 column chromatography, it was eluted from the column as multiple peaks larger than 8 glucose units (Figure 7A). After digestion with *A. saitoi* α-mannosidase I, the oligosaccharides in Figure 7A were separated into two peaks c and d, the former of which was eluted at the same position as authentic Man$_5$·GlcNAc·GlcNAc$_{OT}$ (Figure 7B). Peak c was further

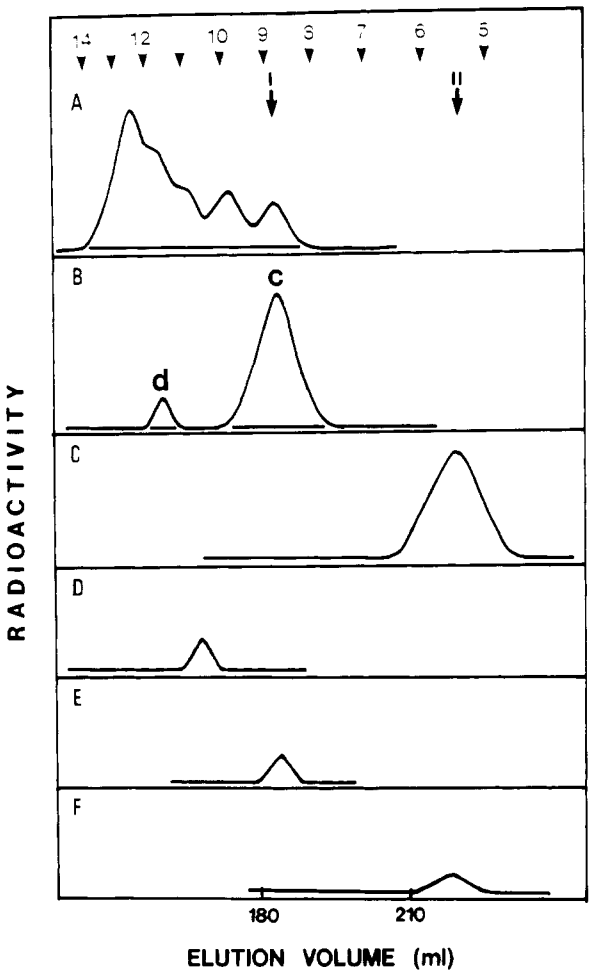
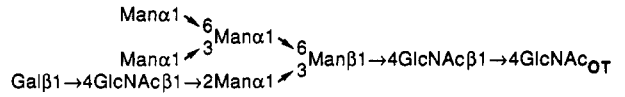


FIGURE 7: Sequential exoglycosidase digestion of fraction N-IV. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL⁻ of fraction N-IV; (B) oligosaccharides indicated by a bar in panel A digested with *A. saitoi* α-mannosidase I; (C) oligosaccharide c in panel B digested with jack bean α-mannosidase; (D) oligosaccharide d in panel B digested with diplococcal β-galactosidase; (E) oligosaccharide in panel D digested with diplococcal β-*N*-acetylhexosaminidase; (F) oligosaccharide in panel E digested with jack bean α-mannosidase. Arrowheads at the top of the figure are the same as in Figure 4. Black arrows indicate the elution positions of authentic oligosaccharides: (I) Man_5·GlcNAc·GlcNAc$_{OT}$; (II) Man·GlcNAc·GlcNAc$_{OT}$.

digested with jack bean α-mannosidase and converted to the product with the same elution position as authentic Man·GlcNAc·GlcNAc$_{OT}$ (Figure 7C). That the product in Figure 7C has the structure Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was confirmed by methods already described. These results indicated that 90% of the oligosaccharides in the fraction AAL⁻ of the fraction N-IV are a series of high mannose-type: $(Man\alpha1\rightarrow2)_{0-4}$·$Man_5$·GlcNAc·GlcNAc$_{OT}$.

When peak d in Figure 7B was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue (Figure 7D) and one *N*-acetylglucosamine residue (Figure 7E) were released. The peak in Figure 7E was digested with jack bean α-mannosidase, and four mannose residues were released (Figure 7F). That the radioactive product in Figure 7F has the structure Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was further confirmed by methods already described. Since methylation analysis showed that oligosaccharide d after digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase contains only 2,3,4,6-tetra-*O*-methyl- and 2,4-di-*O*-methylmannitols as the methylated mannitols in the molar ratio of 3:2, the structure of the peak in Figure 7E can be written either as Manα1→6(Manα1→3)Manα1→6-(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ or as Manα1→6(Manα1→3)Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$. In order to determine which of the structures is the correct one, the radioactive product in Figure 7E was subjected to periodate oxidation, and the product was analyzed by Bio-Gel P-4 column chromatography. A radioactive peak with the same mobility as authentic Manα1→6Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ was obtained (data not shown). This radioactive tetrasaccharide was then subjected to a second periodate oxidation. Analysis of the radioactive product by Bio-Gel P-4 column chromatography revealed that a single radioactive peak corresponding to GlcNAcβ1→4XylNAc$_{OT}$ was obtained (data not shown). These results indicated that the radioactive tetrasaccharide obtained by the first periodate oxidation was not Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ but was Manα1→6Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$. Therefore, the structure of the peak in Figure 7E should be Manα1→6-(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$, because Manα1→6(Manα1→3)Manα1→3-(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ must be converted to Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ by the first periodate oxidation. These results indicated that the component d in Figure 7B is a typical hybrid-type sugar chain and has the following structure:



In contrast, oligosaccharides in fraction AAL⁻ of fraction AN-IV were separated into two components e and f with effective sizes of 12.2 and 13.2 glucose units by Bio-Gel P-4 column chromatography (Figure 8A). When peaks e and f in Figure 8A were separately digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue (solid and dotted lines in Figure 8B, respectively) and one *N*-acetylglucosamine residue (solid and dotted lines in Figure 8C, respectively) were released from both peaks. The dotted line peak with an effective size of 9.9 glucose units in Figure 8C was digested with *A. saitoi* α-mannosidase I, and the product was eluted at 8.9 glucose units (Figure 8D) indicating that the one α1→2 linked mannose residue was released. When the solid line product in Figure 8C and the dotted line product in Figure 8D were digested with jack bean α-mannosidase, four mannose residues were released from both peaks (solid and dotted lines in Figure 8E, respectively). That the solid and dotted line products in Figure 8E have the structure Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was confirmed by methods already described. The elution profiles of oligosaccharides in fraction AAL⁺ of fraction N-IV from a Bio-Gel P-4 column were very similar to those of fraction AAL⁻ of fraction AN-IV, except that the elution position of each peak was larger than the corresponding peak in the AAL⁻ fraction by one glucose unit (data not shown), reflecting the presence of the fucose residue linked to the proximal *N*-acetylglucosamine residue. However, methylation analysis of oligosaccharide f failed to detect 3,4,6-tri-*O*-methylmannitol in their methylated sugars due to the small amount available. Structural analysis of the oligosaccharides in fraction AAL⁺ of fraction AN-IV was not performed due to the trace amount available.

These results indicated that oligosaccharides in fraction AAL⁻ of fraction AN-IV and in fraction AAL⁺ of fraction

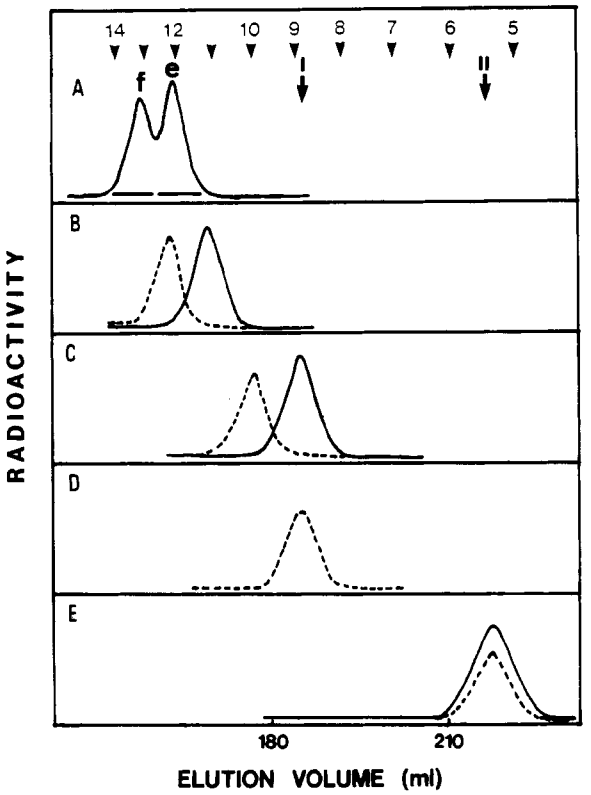
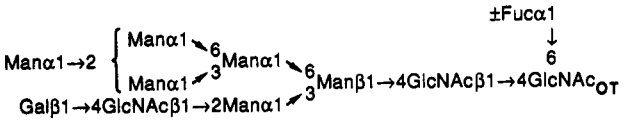


FIGURE 8: Sequential exoglycosidase digestion of fraction AN-IV. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography; (A) fraction AAL^- of fraction AN-IV; (B) solid and dotted lines indicate the oligosaccharides e and f in panel A digested with diplococcal β-galactosidase, respectively; (C) solid and dotted lines indicate the oligosaccharides shown by solid and dotted lines in panel B digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (D) dotted line in panel C digested with *A. saitoi* α-mannosidase I; (E) solid and dotted lines indicate the oligosaccharides shown by solid line in panel C and dotted line in panel D digested with jack bean α-mannosidase, respectively. Arrowheads at the top of the figure are the same as in Figure 4. Black arrows I and II are the same as in Figure 7.

N-IV are hybrid-type sugar chains with and without the fucose attached to the proximal *N*-acetylglucosamine residue, and that about 50% of these hybrid-type sugar chains (0.7% of the total oligosaccharides) contain an additional mannose linked via $\alpha1\rightarrow2$ to one of the nonreducing terminal mannose residues of the sugar chains whose structures can be written as follows:



Structures of Oligosaccharides in Fraction V. Oligosaccharides in fraction AAL^+ of fraction N-V (WFA^+·DSA^-·RCA^-), which contained 5.8% of the total oligosaccharides, were eluted from a Bio-Gel P-4 column as two peaks g and h with effective sizes of 13.8 and 16.0 glucose units (Figure 9A). When peak g in Figure 9A was digested with jack bean β-*N*-acetylhexosaminidase, the product was eluted at the position of 9.8 glucose units, releasing two *N*-acetylhexosamine residues (solid line in Figure 9B). Since oligosaccharide g in Figure 9A was retarded in a Con A–Sepharose column (data not shown), as were oligosaccharides in fraction II, it was assumed to be a hybrid-type sugar chain with the Man$\alpha1\rightarrow2$Man$\alpha1\rightarrow3$Man$\alpha1\rightarrow6$Man group. Accordingly, the solid line peak in Figure 9B was digested with *A. saitoi* α-mannosidase I. One mannose residue

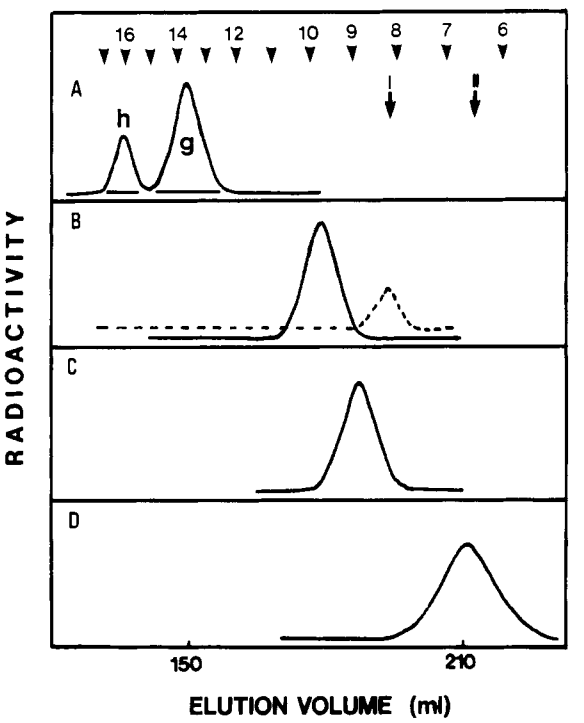


FIGURE 9: Sequential exoglycosidase digestion of fraction N-V. The fraction and its exoglycosidase digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction N-V; (B) solid and dotted lines indicate oligosaccharides g and h digested with jack bean β-*N*-acetylhexosaminidase, respectively; (C) solid line oligosaccharide in panel B digested with *A. saitoi* α-mannosidase I; (D) the oligosaccharide in panel C digested with jack bean α-mannosidase. The arrowheads at the top of the figure are the same as in Figure 4. Black arrows indicate the elution positions of authentic oligosaccharides: (I) Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; (II) Man·GlcNAc·Fuc·$GlcNAc_{OT}$.

was released by the enzymatic treatment (Figure 9C). After digestion of the peak in Figure 9C with jack bean α-mannosidase, three mannose residues were released, and the product was eluted at the same position as authentic Man·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 9D). That the product has the structure Man$\beta1\rightarrow4$GlcNAc$\beta1\rightarrow4$(Fuc$\alpha1\rightarrow6$)-$GlcNAc_{OT}$ was confirmed by methods already described.

Oligosaccharide h in Figure 9A bound to a Con A–Sepharose column and was eluted from the column with 5 mM α-methyl D-glucopyranoside, indicating that it is a biantennary complex-type sugar chain. When oligosaccharide h in Figure 9A was digested with jack bean β-*N*-acetylhexosaminidase, four *N*-acetylhexosamine residues were released, and the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (dotted line in Figure 9B). That the product has the structure Man$\alpha1\rightarrow6$(Man$\alpha1\rightarrow3$)Man-$\beta1\rightarrow4$GlcNAc$\beta1\rightarrow4$(Fuc$\alpha1\rightarrow6$)$GlcNAc_{OT}$ was confirmed by methods already described.

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL^+ of fraction AN-V gave the same series of results as described earlier for the components g and h in Figure 9A (data not shown). Similarly, two peaks g′ and h′, which corresponded to peaks g and h in Figure 9A, were obtained from fraction AAL^- of fractions AN-V and N-V by Bio-Gel P-4 column chromatography. The elution profiles of oligosaccharides g′ and h′ and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of oligosaccharides g and h in fraction AAL^+ of fraction N-V, except that the elution positions of each peak were smaller than the corresponding peaks in fraction AAL^+ by one glucose unit (data not shown). These results indicated

Table III: Methylation Analysis of Each Lectin Fraction Obtained from a Mixture of Fractions N and AN

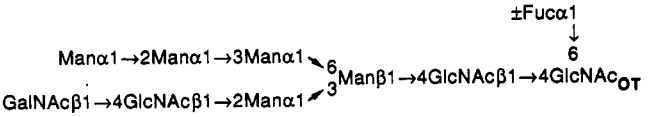
	fractions, molar ratio[a]								
	WFA$^+$·DSA$^-$·RCA$^-$(V)					WFA$^+$·DSA$^-$·RCA^r(VI)		WFA$^+$·DSA$^+$(VII)	
partially *O*-methylated sugars	(g+g′)	(g+g′)[b]	(g+g′)[c]	(h+h′)	(h+h′)[c]	(i+i′)	(i+i′)[c]	(j+j′)	(k+k′)
fucitol									
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.6	0.5	0.6	0.7	0.6	0.7	0.7	0.8	0.7
galactitol									
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	–[d]	–	–	–	–	0.9	0.8	1.7	2.7
mannitol									
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.7	1.8	–	1.9	–	1.7	–	–
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	1.8	0.9	0.9	1.9	–	1.8	–	0.8	–
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0.7	0.7	0.8	–	–	–	–	–	–
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	–	–	–	–	–	–	–	–	0.8
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	–	–	–	–	–	–	–	0.8	0.7
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2-*N*-methylacetamido-2-deoxyglucitol									
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	1.7	1.7	0.8	2.7	0.8	2.6	0.8	3.5	4.4
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.2
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.7	0.6	0.7	0.7	0.6	0.6	0.6	0.7	0.7
2-*N*-methylacetamido-2-deoxygalactitol									
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.7	–	1.7	–	0.8	–	1.1	1.0

[a] Numbers in the table were calculated by taking the value of 2,4-di-*O*-methyl mannitol as 1.0. [b] After *A. saitoi* α-mannosidase I digestion. [c] After jack bean β-*N*-acetylhexosaminidase digestion. [d] Not detectable.

that the structures of oligosaccharides in fraction AAL$^-$ contain nonfucosylated trimannosyl cores.

Comparison of the methylation data of combined oligosaccharides g+g′ before and after digestion with jack bean β-*N*-acetylhexosaminidase indicated that an *N*-acetylgalactosamine residue is linked at the C-4 position of the *N*-acetylglucosamine residue of the GlcNAcβ1→2Man group. Decreases of approximately 1 mol each of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol, 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol, and 3,4,6-tri-*O*-methylmannitol and an increase of 1 mol of 2,3,4,6-tetra-*O*-methylmannitol were obtained after the enzyme digestion (Table III). Therefore, the data indicated that oligosaccharides g+g′ contain the GalNAcl→4GlcNAcl→2Man structure. Similarly, comparison of the methylation data of oligosaccharides g+g′ before and after digestion with *A. saitoi* α-mannosidase I showed a decrease of approximately 1 mol of 3,4,6-tri-*O*-methylmannitol after the enzyme digestion. However, an equivalent amount of 2,3,4,6-tetra-*O*-methylmannitol was detected before and after the enzyme digestion (Table III), indicating that a mannose residue is linked at the C-2 position of another mannose residue. In order to determine to which arm of the trimannosyl core the GalNAcβ1→4GlcNAc group is linked, oligosaccharide g and g′ before and after digestion with jack bean β-*N*-acetylhexosaminidase were treated with endo D. Both oligosaccharides were resistant to endo D digestion before jack bean β-*N*-acetylhexosaminidase digestion, while the de-*N*-acetylgalactosaminylated and de-*N*-acetylglucosaminylated oligosaccharides were completely hydrolyzed by endo D and liberated radioactive *N*-acetylglucosaminitol and fucosyl *N*-acetylglucosaminitol, respectively (data not shown). Since endo D requires the general structure of R→Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc (in which R represents either hydrogen or sugars) for its substrate as described earlier, the results indicated that the GalNAcβ1→4GlcNAc group should be linked to the Manα1→3 arm. On the other hand, oligosaccharides g and g′ were completely hydrolyzed by digestion with endo H and liberated radioactive *N*-acetylglucosaminitol and fucosyl *N*-acetylglucosaminitol, respectively (data not shown). Since this endo H requires the general structure of R_1→6(R_2→Manα1→3)Manα1→6(R_3→Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc (in which R_1, R_2 and R_3 represent either hydrogen or sugars) for its substrate as also described earlier, the results indicated that the Manα1→2Manα1→3Man group should be linked to the Manα1→6 arm.

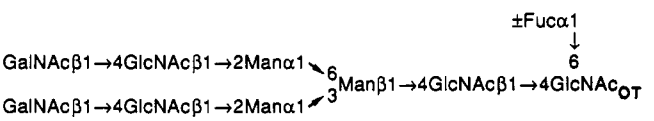
These results together with the sequential exoglycosidase digestion data already described indicated that oligosaccharides g and g′ have the following structures.



Digestion of oligosaccharides in fractions AAL$^-$ and AAL$^+$ of fraction AN-V with endo D and endo H gave the same results as those described earlier for oligosaccharides g and g′ (data not shown).

Similarly, comparison of the methylation data of combined oligosaccharides h+h′ before and after digestion with jack bean β-*N*-acetylhexosaminidase indicated that two GalNAcl→4GlcNAcl→2Man groups are present in each oligosaccharide, because approximately 2 mol each of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol, 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol, and 3,4,6-tri-*O*-methylmannitol vanished and 2 mol of 2,3,4,6-tetra-*O*-methylmannitol appeared after the enzyme digestion (Table III).

These results together with the sequential exoglycosidase digestion data already described indicated that oligosaccharides h+h′ have the following structures.



Structures of Oligosaccharides in Fraction VI. Since oligosaccharides in fraction VI bound to a WFA-agarose column and were retarded in an RCA-I–agarose column, each oligosaccharide in this fraction was considered to have at least one *N*-acetylgalactosamine residue and one β-galactose residue at their nonreducing termini. Oligosaccharide in fraction AAL$^+$ of fraction AN-VI, which contained 5.7% of the total

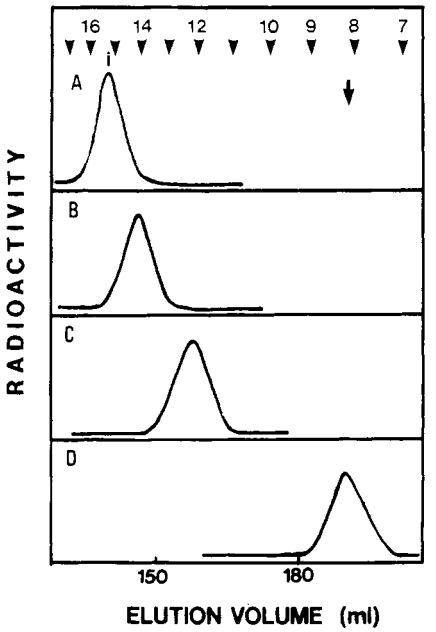
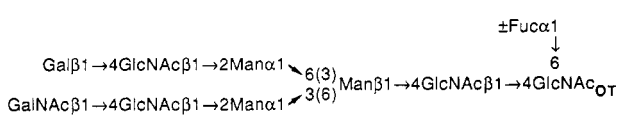


FIGURE 10: Sequential exoglycosidase digestion of fraction AN-VI. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction AN-VI; (B) oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) oligosaccharide in panel B digested with diplococcal β-*N*-acetylhexosaminidase; (D) oligosaccharide in panel C digested with jack bean β-*N*-acetylhexosaminidase. Arrowheads at the top of the figure and the black arrow are the same as in Figure 4.

oligosaccharides, was eluted from a Bio-Gel P-4 column as a single peak (named component i) with an effective size of 15.2 glucose units (Figure 10A). When component i in Figure 10A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose (Figure 10B) and one *N*-acetylglucosamine residue (Figure 10C) were released. When the peak in Figure 10C with an effective size of 12.2 glucose units was digested with jack bean β-*N*-acetylhexosaminidase, the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ with an effective size of 8.2 glucose units, indicating that two *N*-acetylhexosamine residues were released (Figure 10D). That the product has the structure: Manα1→6(Manα1→3)-Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by methods already described.

Sequential exoglycosidase digestion of oligosaccharide in fraction AAL^+ of fraction N-VI gave the same series of results as described earlier for component i in Figure 10A. The elution profiles of oligosaccharides in fraction AAL^- of fractions AN-VI and N-VI and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL^+ of fraction AN-VI, except that the elution position of each peak was smaller than the corresponding peak in AAL^+ fraction by one glucose unit (data not shown).

The binding specificities of the immobilized lectin columns used for fractionation and the methylation data of the oligosaccharides in this fraction before and after digestion with jack bean β-*N*-acetylhexosaminidase (Table III) indicated that the oligosaccharides in this fraction have the following structures.



In order to determine the location of the GalNAcβ1→4GlcNAc group, the oligosaccharides in fraction VI digested with jack bean β-*N*-acetylhexosaminidase were applied to a Con A–Sepharose column. All oligosaccharides bound to the column, and 43% of the bound oligosaccharides was eluted from the column with 5 mM α-methyl D-glucopyranoside, and the remaining 57% was eluted with 100 mM α-methyl D-mannopyranoside (data not shown). Since Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OT}$ and Galβ1→4GlcNAcβ1→2Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OT}$ bind to a Con A–Sepharose column and are eluted with 5 mM α-methyl D-glucopyranoside and 100 mM α-methyl D-mannopyranoside, respectively (Yamashita et al., 1981; Merkle & Cummings, 1987), the GalNAcβ1→4GlcNAc group of oligosaccharides in fraction VI should be linked almost evenly to both α-mannosyl arms of the trimannosyl core.

Structures of Oligosaccharides in Fraction VII. Oligosaccharides in fraction AAL^+ of fraction AN-VII, which contained 1.3% of the total oligosaccharides, were eluted from a Bio-Gel P-4 column as two peaks j and k (Figure 11A). When peak j in Figure 11A was digested with jack bean β-*N*-acetylhexosaminidase, two products l and m were obtained, releasing four and two β-*N*-acetylhexosamine residues, respectively (Figure 11B). After digestion of peak l in Figure 11B with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose (solid line peak in Figure 11C) and one *N*-acetylglucosamine (solid line in Figure 11E) were released. When peak m in Figure 11B was digested with diplococcal β-galactosidase, two galactose residues were released (dotted line in Figure 11C). The dotted line peak in Figure 11C was converted to three peaks n, o, and p by digestion with diplococcal β-*N*-acetylhexosaminidase, releasing two, one, and zero *N*-acetylglucosamine residue, respectively (Figure 11D). When peaks o and p in Figure 11D were digested with jack bean β-*N*-acetylhexosaminidase, one and two *N*-acetylglucosamine residues were released, and both products were eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (dotted and dot-dashed lines in Figure 11E, respectively). That peak n in Figure 11D and the solid, dotted, and dot-dashed line products in Figure 11E have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)-$GlcNAc_{OT}$ was confirmed by methods already described. Therefore, these results together with methylation data of the oligosaccharides before and after digestion with jack bean β-*N*-acetylhexosaminidase (data not shown) indicated that component j in Figure 11A is a mixture of 2,6-branched triantennary complex-type sugar chains with one and two GalNAcβ1→4GlcNAc groups. Because 3,6-di-*O*-methylmannitol was not detected in this oligosaccharide fraction by methylation analysis (Table III), the oligosaccharide does not contain the GlcNAcβ1→4(GlcNAcβ1→2)Man group. Since the dotted line peak in Figure 11C was separated into three peaks by digestion with diplococcal β-*N*-acetylhexosaminidase, which cleaves the GlcNAcβ1→2Man linkage but not that of the GlcNAcβ1→6(GlcNAcβ1→2)Man group (Figure 11D), peak n is considered to be derived from the oligosaccharide with the GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Man group, peak o from the oligosaccharide with the GlcNAcβ1→6Manα1→6 or 3(GlcNAcβ1→2Manα1→3 or 6)Man group, and peak p from the oligosaccharide with the GlcNAcβ1→6(GlcNAcβ1→2)Man group. Therefore, peak j in Figure 11A contains three isomeric triantennary complex-type oligosaccharides in which the

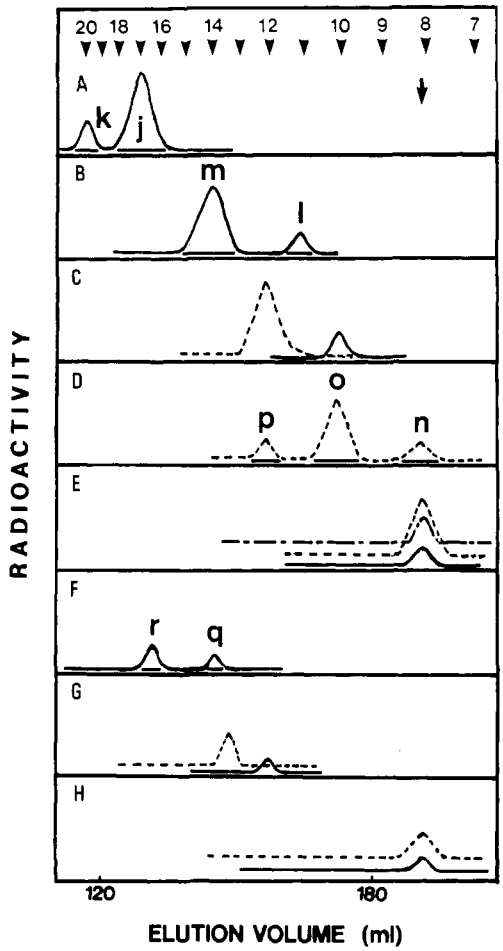


FIGURE 11: Sequential exoglycosidase digestion of fraction AN-VII. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography. (A) fraction AAL^+ of fraction AN-VII; (B) peak j digested with jack bean β-*N*-acetylhexosaminidase; (C) solid and dotted lines indicate oligosaccharides l and m in panel B digested with diplococcal β-galactosidase; (D) oligosaccharide shown by dotted line in panel C digested with diplococcal β-*N*-acetylhexosaminidase; (E) solid, dotted, and dot-dashed lines indicate the oligosaccharide shown by solid line in panel C, and oligosaccharides o and p in panel D, digested with jack bean β-*N*-acetylhexosaminidase, respectively; (F) peak k in panel A digested with jack bean β-*N*-acetylhexosaminidase; (G) solid and dotted lines indicate oligosaccharides q and r in panel F digested with diplococcal β-galactosidase, respectively; (H) solid and dotted lines indicate the oligosaccharides shown by solid and dotted lines in panel G digested with jack bean β-*N*-acetylhexosaminidase, respectively. The arrowheads at the top of the figure and the black arrow are the same as in Figure 4.

GalNAcβ1→4GlcNAc group is present on every one of three outer chains, and the frequency of *N*-acetylgalactosaminylation of the GlcNAcβ1→2Man group of the GlcNAcβ1→6(GlcNAcβ1→2)Man branch is approximately twice of that of the other branches.

After digestion with jack bean β-*N*-acetylhexosaminidase, component k in Figure 11A was eluted from a Bio-Gel P-4 column as two peaks q and r, releasing four and two *N*-acetylhexosamine residues, respectively (Figure 11F). The result suggested that peak k in Figure 11A is also a mixture of oligosaccharides with one and two GalNAcβ1→4GlcNAc groups. When peak q was digested with diplococcal β-galactosidase followed by jack bean β-*N*-acetylhexosaminidase, two galactose residues (solid line in Figure 11G) and two *N*-acetylglucosamine residues were released, and the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (solid line in Figure 11H). Similarly, when peak r in Figure 11F was digested with diplococcal β-galactosidase followed by jack bean β-*N*-acetylhexosaminidase, three galactose residues (dotted line in Figure 11G) and three *N*-acetylglucosamine residues were released, and the product was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (dotted line in Figure 11H). That the solid and dotted line products in Figure 11H have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by methods already described.

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL^+ of fraction N-VII gave the same series of results as described earlier for the components in Figure 11A. The elution profiles of oligosaccharides in fraction AAL^- of fractions AN-VII and N-VII and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL^+ of fraction AN-VII, except that the elution position of each peak was smaller than the corresponding peak in the AAL^+ fraction by one glucose unit (data not shown).

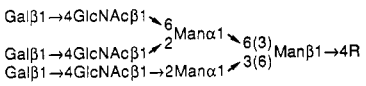
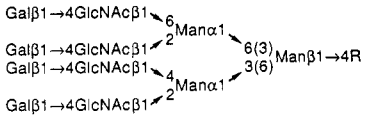
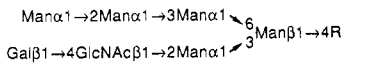
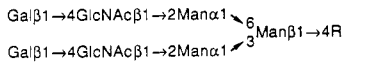
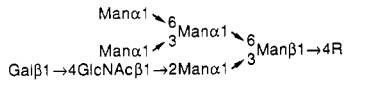
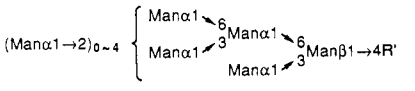
Comparison of the methylation data of combined oligosaccharides k+k′ (k′ corresponded to k, obtained from the fraction AAL^-) before and after digestion with jack bean β-*N*-acetylhexosaminidase indicated that at least one GalNAcβ1→4GlcNAc group is present in each oligosaccharide, because approximately 1 mol each of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol and 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol vanished and approximately 1 mol of 2,3,4,6-tetra-*O*-methylmannitol appeared after the enzyme digestion (data not shown).

These results together with the exoglycosidase digestion data already described indicated that component k in Figure 11A is a mixture of tetraantennary complex-type sugar chains with one and two GalNAcβ1→4GlcNAc groups. Due to a small amount of component k in Figure 11A, the location of the GalNAcβ1→4GlcNAc group in the outer branches of the sugar chains could not be determined.

Methylation Analysis of Oligosaccharides. In order to confirm each glycosidic linkage in the oligosaccharides of CD36 as determined mainly by sequential exoglycosidase digestion, deuterium-labeled oligosaccharides in fractions N and AN were mixed and subjected to serial lectin column chromatography similar to that described in Table I prior to methylation analysis. Briefly, oligosaccharides that do not have the GalNAcβ1→4GlcNAc group were separated into the WFA^-·Con A^-, WFA^-·Con A^r, WFA^-·Con A^+, and WFA^-·Con A^{++} fractions which corresponded to fractions I, II, III, and IV as described in Table I. Similarly, oligosaccharides that have the GalNAcβ1→4GlcNAc group were separated into the WFA^+·DSA^-·RCA-I^-, WFA^+·DSA^-·RCA-I^r, and WFA^+·DSA^+ fractions which corresponded to fractions V, VI, and VII as described in Table I. After the haptenic monosaccharides used for eluting the bound materials were removed by Bio-Gel P-4 column chromatography, each fraction except for fraction WFA^+·DSA^+ was subjected to methylation analysis. Since the fraction WFA^+·DSA^+ contained 1% *N*-acetylglucosamine oligomers which cannot be removed by Bio-Gel P-4 column chromatography, the haptenic sugars were removed by chromatography on a WFA–agarose column, followed by a Bio-Gel P-4 column.

In order to confirm the presence and amounts of *N*-acetylgalactosamine, combined oligosaccharides g+g′ and h+h′ were prepared by isolating g and h from fraction AAL^+ and g′ and h′ from fraction AAL^- of fraction WFA^+·DSA^-·RCA-I^- on a Bio-Gel P-4 column (g′ and h′ corresponded to g and h in Figure 9A, respectively). Combined oligosaccharides i+i′,

Table IV: Proposed Structures of the Neutral and Desialylated Asn-Linked Sugar Chains of Bovine MFGM CD36

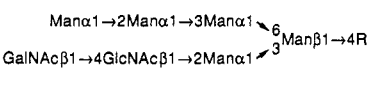
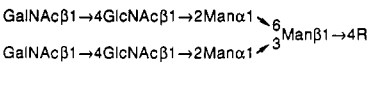
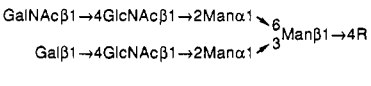
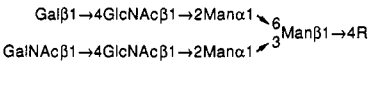
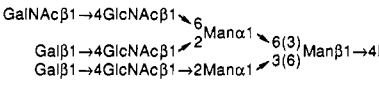
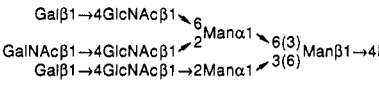
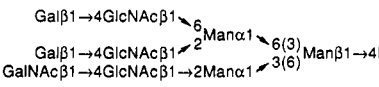
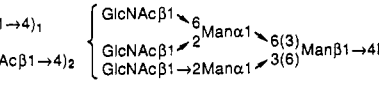
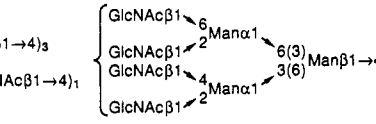
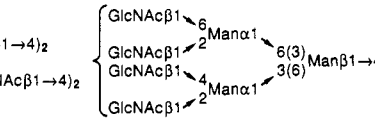
no.[a]	components[b]	structures[c]	molar ratio (%)
I.	a		7.2
I.	b		1.6
II.			11.6
III.			15.6
IV.	e		4.9
IV.			30.1

[a] Fractions I–IV shown in Table I. [b] Oligosaccharide components shown in Figures 4 and 8. [c] R = GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. R′ = GlcNAcβ1→4GlcNAc$_{OT}$.

j+j′ and k+k′ were also prepared by isolating i, j, and k from fraction AAL$^+$ and i′, j′, and k′ from fraction AAL$^-$ of fractions WFA$^+$·DSA$^-$·RCA-I^r and WFA$^+$·DSA$^+$ (i′, and j′ and k′ corresponded to i, and j and k in Figures 10A and 11A, respectively). The five oligosaccharide fractions thus prepared were subjected to methylation analysis with use of GM_2-ganglioside, GalNAcβ1→4(Neu5Acα2→3)Galβ1→4Glcβ1→ceramide, as a standard. Furthermore, in order to confirm the structure of the GalNAcβ1→4GlcNAcβ1→2Man group, combined oligosaccharides g+g′, h+h′, i+i′, j+j′, and k+k′ were subjected to methylation analysis before and after digestion with jack bean β-*N*-acetylhexosaminidase. In the case of fraction WFA$^-$·Con A^r and oligosaccharides g+g′ in fraction WFA$^+$·DSA$^-$·RCA-I$^-$, methylation analysis was also performed before and after digestion with *A. saitoi* α-mannosidase I in order to confirm the structure of the Manα1→2Man group.

As shown in Tables II and III, the molar ratio of each methylated sugar was calculated by taking the value of 2,4-di-*O*-methylmannitol as 1.0, except for fraction WFA$^-$·Con A^{++} which was calculated by taking the value of 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol as 1.0. Comparison of the data of fraction WFA$^-$·Con A^r (Table II) and oligosaccharides g+g′ (Table III), before and after digestion with α-mannosidase I, indicated that a mannose residue is linked at the C-2 position of another mannose residue, because the amount of 3,4,6-tri-*O*-methylmannitol was reduced to a half of that after the enzyme digestion, but the equivalent amount of 2,3,4,6-tetra-*O*-methylmannitol was present before and after the enzyme digestion. The methylation data of fractions WFA$^-$·Con A$^-$, WFA$^-$·Con A^r, WFA$^-$·Con A$^+$, and WFA$^-$·Con A^{++} (Table II) indicated that oligosaccharides in the above fractions are of tri- and tetraantennary complex-type, hybrid-type, biantennary complex-type, and high mannose-type, respectively.

Table V: Proposed Structures of the Neutral and Desialylated Asn-Linked Sugar Chains of Bovine MFGM CD36

no.[a]	components[b]	structures[c]	molar ratio (%)
V.	g		7.7
V.	h		4.1
VI.	i		5.7
VI.	i		7.6
VII.	j		0.5
VII.	j		1.1
VII.	j		0.6
VII.	j		0.4
VII.	k		0.5
VII.	k		0.1

[a] Fractions V–VII shown in Table I. [b] Oligosaccharide components shown in Figures 9, 10, and 11. [c] R = GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$.

Detection of 0.7–1.7 mol of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol in fractions WFA$^+$·DSA$^-$·RCA-I$^-$, WFA$^+$·DSA$^-$·RCA-I^r, and WFA$^+$·DSA$^+$ (Table III) indicated that the oligosaccharides in each fraction contain 1–2 mol of *N*-acetylgalactosamine at their nonreducing termini. Since 1 mol each of 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol, 3,6-di-*O*-methyl-2-*N*-methylacetamido-2-deoxyglucitol, and 3,4,6-tri-*O*-methylmannitol vanished in the methylated sugars derived from combined oligosaccharides g+g′, h+h′, i+i′ (Table III), and j+j′ and k+k′ (data not shown) after digestion with jack bean β-*N*-acetylhexosaminidase, these oligosaccharides should contain the structure of the GalNAcl→4GlcNAcl→2Man group. The methylation data of fractions WFA$^+$·DSA$^-$·RCA-I$^-$, WFA$^+$·DSA$^-$·RCA-I^r and WFA$^+$·DSA$^+$ (Table III) indicated that these fractions contain hybrid-type and biantennary complex-type, biantennary complex-type, and tri- and tetraantennary complex-type sugar chains.

The proposed oligosaccharide structures of bovine MFGM CD36, as determined by sequential exoglycosidase digestion

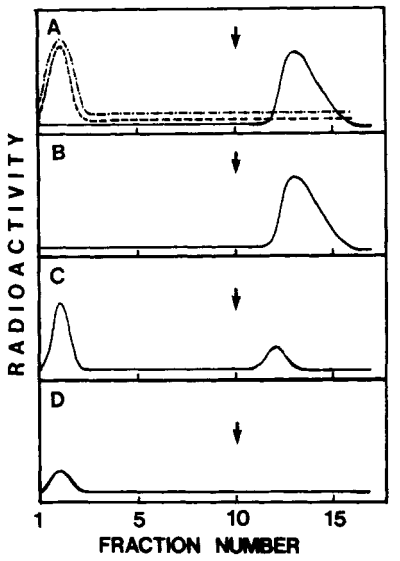
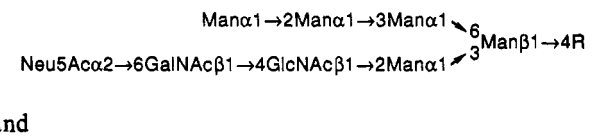


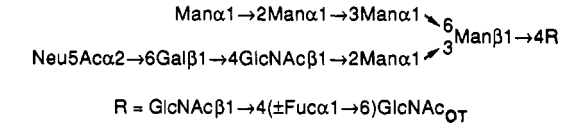
FIGURE 12: Affinity chromatography of hybrid-type oligosaccharides in fraction A on SNA–agarose (A and B) and WFA–agarose (C and D) columns. Panel A shows the elution profiles of authentic oligosaccharides from an SNA–agarose column: solid line, Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ prepared from human transferrin; dotted line, Neu5Acα2→3Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→3Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ from human placental alkaline phosphatase; dot-dashed line, Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$. After separation of the sialylated oligosaccharides by Con A–Sepharose column chromatography, oligosaccharides which were retarded in the column were subjected to SNA–agarose column chromatography (B). The bound oligosaccharides in panel B after exhaustive digestion with *A. ureafaciens* sialidase were applied to a WFA–agarose column (C). The bound oligosaccharides in panel C after digestion with jack bean β-*N*-acetylhexosaminidase were applied to a WFA–agarose column (D).

and methylation analysis, are summarized in Tables IV and V.

Structure of the Sialylated GalNAcβ1→4GlcNAc Group. Since hybrid-type sugar chains with the GalNAcβ1→4GlcNAc group were detected in fraction AN, the GalNAcβ1→4GlcNAc group of the sugar chains as well as those of the complex-type sugar chains were assumed to be sialylated. In order to determine the sialylated structure, oligosaccharides in fraction A were chromatographed on a Con A–Sepharose column. The hybrid-type sugar chains with the Manα1→2Manα1→3Manα1→6Man outer chain, which were recovered from the retarded fraction of the column, were then applied to a SNA–agarose column. SNA is known to bind the sialylated oligosaccharides with the Neu5Acα2→6Gal/GalNAc group but not with the Neu5Acα2→3Gal/GalNAc group or the Galβ1→3(Neu5Acα2→6)GlcNAc group (Shibuya et al., 1987a,b). Consistent with the binding specificity of this lectin, a biantennary complex-type sugar chain with the Neu5Acα2→6Gal group prepared from human transferrin (Spik et al., 1975) bound and was eluted from the column with 100 mM lactose (solid line in Figure 12A), but the complex-type sugar chains with the Neu5Acα2→3Gal group prepared from human placental alkaline phosphatase (Endo et al., 1988) did not bind (dashed line in Figure 12A). In addition, the desialylated human transferrin sugar chain no longer bound to a SNA–agarose column (dot-dashed line in Figure 12A). Under the same conditions as above, all of the hybrid-type sugar chains (Con A^r fraction of fraction A) bound and were eluted from the column with 100 mM lactose (Figure 12B), indicating that the sialylated hybrid-type sugar chains from CD36 contain only the Neu5Acα2→6Gal/GalNAc group. The sialylated hybrid-type sugar chains were then digested exhaustively with *A. ureafaciens* sialidase, and the desialylated oligosaccharides were applied to a WFA–agarose column. The result showed that 20% of the applied oligosaccharides was bound and eluted from the column with 100 mM *N*-acetylgalactosamine (Figure 12C). Since the sialylated oligosaccharides did not bind to a WFA–agarose column, sialic acid was assumed to be linked to the penultimate *N*-acetylgalactosamine residue. After digestion of the oligosaccharides which bound to a WFA–agarose column with jack bean β-*N*-acetylhexosaminidase, they no longer bound to a WFA–agarose column (Figure 12D). In contrast, the oligosaccharides that passed through a WFA–agarose column were all retarded in an RCA-I–agarose column (data not shown), indicating that these sugar chains are terminated with β-galactose residues. For further confirmation of the sialylated outer chain structures, substantial amounts of the sialylated and desialylated hybrid-type sugar chains of CD36 were isolated and subjected to methylation analysis. The results showed that 2,3,4-tri-*O*-methylgalactitol and 3,4-di-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol are present at the ratio of 4:1 in the sialylated sugar chains (data not shown) but not in the desialylated sugar chains, which gave 2,3,4,6-tetra-*O*-methylgalactitol and 3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol (Tables II and III). These results indicated that the sialylated hybrid-type sugar chains from CD36 that were retarded in a Con A–Sepharose column have the following structures:



and



R = GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$

Although some of the complex-type sugar chains with the GalNAcβ1→4GlcNAc group(s) from CD36 were sialylated, whether these sugar chains contain the Neu5Acα2→6GalNAcβ1→4GlcNAc group or not remains to be elucidated.

DISCUSSION

On the basis of the results presented here, bovine MFGM CD36 was shown to contain six asparagine-linked sugar chains per molecule, consisting of high mannose-type, hybrid-type, and bi-, tri-, and tetraantennary complex-type sugar chains. Quite interestingly, about 28% of the total oligosaccharides obtained from CD36 was shown to contain the GalNAcβ1→4GlcNAc group(s). The GalNAcβ1→4GlcNAc group was found in bi-, tri-, and tetraantennary complex-type and hybrid-type sugar chains. Since the tri- and tetraantennary complex-type sugar chains with the GalNAcβ1→4GlcNAc groups bound to a DSA–Sepharose column, it was concluded that *N*-acetylgalactosamine can replace the β-galactosyl residues in the oligosaccharide structure recognized by DSA (Yamashita et al., 1987). Distribution of the asparagine-linked sugar chains containing the GalNAcβ1→4GlcNAc group in their outer chain moieties is limited to a small number of glycoproteins. They are the mammalian pituitary glycohormones which contain biantennary complex-type and hybrid-

type sugar chains with the sulfated GalNAcβ1→4GlcNAc group (Green & Baenziger, 1988a,b) and glycoproteins synthesized by the blood fluke *Schistosoma mansoni* adult male whose sugar chains terminate with β-*N*-acetylgalactosamine residues (Nyame et al., 1989; Srivatsan et al., 1992). In contrast, bovine MFGM CD36 sugar chains contained the Neu5Acα2→6GalNAcβ1→4GlcNAc group, but not a sulfated group. The sialylated form of this group has been found recently in the sugar chains of human luteinizing hormone (Weisshaar et al., 1991) and tissue plasminogen activator produced by Bowes melanoma cells (Chan et al., 1991).

It has been reported that the *N*-acetylgalactosaminylation of glycoprotein sugar chains is catalyzed by the peptide specific β-*N*-acetylgalactosaminyltransferase which recognizes the Pro-Xaa-Arg/Lys sequence located 6–9 amino acid residues distant from the N-terminus of putative glycosylation sites (Smith & Baenziger, 1992). Whether bovine MFGM CD36 includes such a tripeptide sequence or bovine mammary epithelial cells contain another β-*N*-acetylgalactosaminyltransferase with different acceptor specificities has to be clarified. Partial protein sequence data show that bovine epithelial cell CD36 does have at least one Pro-Xaa-Arg sequence beginning at amino acid residue 395. However, it is 18 residues distant from a putative glycosylation site (Greenwalt, unpublished data).

Another interesting point is that most of the hybrid-type sugar chains obtained from CD36 was shown to contain the Manα1→2Manα1→3Manα1→6Man group in addition to a small amount of typical hybrid-type sugar chains with the Manα1→6(Manα1→3)Manα1→6Man group. The major pathway of asparagine-linked sugar chain biosynthesis after removal of three glucose residues from the tetradecasaccharide Glc_3·Man_9·$GlcNAc_2$ involves release of all four α-1,2-linked mannose residues by distinct mannosidases included in the endoplasmic reticulum and Golgi apparatus. The resulting protein-linked heptasaccharide Man_5·$GlcNAc_2$-Asn-peptide is the preferred substrate for *N*-acetylglucosaminyltransferase I. After this reaction, the hybrid-type product GlcNAc·Man_5·$GlcNAc_2$-Asn-peptide is hydrolyzed by Golgi α-mannosidase II, which releases α-1,6-linked and α-1,3-linked mannose residues, and converted to complex-type sugar chains. The hybrid-type sugar chains with the Manα1→2Manα1→3Manα1→6Man group found in the CD36 reported here do not meet with this biosynthetic pathway and suggest that the bovine mammary epithelial cells have a different processing pathway for asparagine-linked sugar chains which contains α-mannosidases and an *N*-acetylglucosaminyltransferase I with different substrate specificities. In support of this hypothesis, several α-1,2-mannosidases which differ from the well known α-1,2-mannosidases in their substrate specificities, their sensitivities to inhibitors such as deoxymannojirimycin and swainsonine, and their kinetic and physical properties have been purified from a variety of animal tissues (Shoup & Touster, 1976; Tabas & Kornfeld, 1979; Forsee & Schutzbach, 1981; Tulsiani et al., 1982; Tulsiani & Touster, 1985; Bischoff & Kornfeld, 1986; Schweden et al., 1986; Forsee et al., 1989; Bonay & Hughes, 1991). These reports suggest that the trimming pathway of high mannose-type oligosaccharides to hybrid-type ones may be distinct in different cell types. The presence of an endo-α-mannosidase in rat liver (Lubas & Spiro, 1987) provides evidence for an additional route in the processing pathway of asparagine-linked sugar chains of mammalian glycoproteins. Thus, it is possible that the high mannose-type oligosaccharide: Man_9·$GlcNAc_2$ in bovine mammary epithelial cells can be processed mainly to oligosaccharides with the ±Manα1→6(Manα1→2Manα1→3)-Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc structure by the α-mannosidases with restricted substrate specificities. Whether an oligosaccharide with the above structure can become an acceptor molecule of *N*-acetylglucosaminyltransferase I or not is another problem to be solved. After the *N*-acetylglucosaminylation, the terminal α-1,6-linked mannose residue can be removed by Golgi α-mannosidase II to generate the novel hybrid-type oligosaccharide structures described in this study. Alternatively, it is possible that a biosynthetic intermediate with the ±Manα1→6(Manα1→3)-Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc structure could be mannosylated by the α-1,2-mannosyltransferase in bovine mammary epithelial cells in a manner similar to glucosylation of peptide-linked high mannose-type oligosaccharides Man_{9-6}·$GlcNAc_2$-Asn-peptide by the soluble glucosyltransferase in the endoplasmic reticulum (Parodi et al., 1983, 1984). Studies of the biosynthetic mechanism of asparagine-linked sugar chains in bovine mammary epithelial cells may unravel a new aspect of glycosylation of proteins.

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