Structures of the Asparagine-Linked Sugar Chain of Glucose Transporter from Human Erythrocytes†

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ABSTRACT: The asparagine-linked sugar chain of glucose transporter from human erythrocytes was quantitatively released as oligosaccharides from the polypeptide backbone by hydrazinolysis. They were converted to radioactive oligosaccharides by NaB^3H_4 reduction after N-acetylation and fractionated by anion-exchange column chromatography and Bio-Gel P-4 column chromatography after sialidase treatment. Structural study of each oligosaccharide by exo- and endoglycosidase digestion and methylation analysis indicated that the glycoprotein contains a high-mannose-type oligosaccharide, Man_9·GlcNAc·GlcNAc, and biantennary complex-type oligosaccharides with Manα1→6(±GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc as their cores and the poly-*N*-acetyllactosamine composed of about 16 *N*-acetyllactosaminyl units as their outer chains. These structural features of the sugar moiety of glucose transporter are quite different from those of two major intrinsic glycoproteins of human erythrocytes, glycophorin A and band 3.

D-Glucose enters into human erythrocytes by a facilitated diffusion process. Much evidence indicates that this process is mediated by a protein named glucose transporter. Because the energy production of erythrocytes depends solely on anaerobic glycolysis, glucose transporter is considered to play an important physiological role for erythrocytes. Kasahara and Hinkle (1976) purified a glucose transporter from human erythrocytes. It is a glycoprotein containing approximately 15% carbohydrate (Kasahara & Hinkle, 1977; Sogin & Hinkle, 1978). The glucose transporting activity was fully expressed by incorporating the purified glucose transporter in liposomes (Kasahara & Hinkle, 1977; Baly & Horuk, 1988).

The complete amino acid sequence of the glucose transporter of human HepG2 hepatocarcinoma cells was proposed from the analysis of a complementary DNA clone (Mueckler et al., 1985). By comparing the data with the amino acid sequence of the purified human erythrocyte glucose transporter, it was concluded that the HepG2 and erythrocyte transporters are highly homologous or may be identical. Analysis of these primary polypeptide structures suggested the presence of two potential glycosylation sites, Asn^{45} and Asn^{411}. Since Asn^{411} is included within a membrane-spanning hydrophobic domain, only Asn^{45} should be actually glycosylated (Mueckler et al., 1985).

Several other glycoproteins were found to occur in human erythrocyte membranes. Structures of the sugar chains of only two major integral glycoproteins, glycophorin A and band 3, were fully investigated by several groups (Yoshima et al., 1980a; Tsuji et al., 1980, 1981; Fukuda et al., 1984b). Interestingly, these two glycoproteins have different sets of oligosaccharides, although they are synthesized by the same cells. Glycophorin A has complex-type biantennary sugar chains with the Neu5Acα2→6Galβ1→4GlcNAc group in their outer-chain moieties (Yoshima et al., 1980a). By contrast, the outer chains of most of the sugar chains of band 3 were poly-*N*-acetyllactosamine units (Tsuji et al., 1980, 1981; Fukuda et al., 1984b). Since the human erythrocyte glucose transporter was sensitive to endo-β-galactosidase digestion, its sugar chains are considered to contain the poly-*N*-acetyllactosamine units as their outer chains (Gorga et al., 1979). Heterogeneous glycosylation of the glucose transporter was suggested (Kasahara & Hinkle, 1977), and approximately 50% of this glycoprotein bound to a *Ricinus communis* agglutinin I–Sepharose column (Gorga et al., 1979). However, actual structures of the sugar chains had not been elucidated.

In this study, we would like to report the complete structures of the asparagine-linked sugar chain of the glucose transporter purified from human erythrocytes and show that they are different from those of band 3 and glycophorin A.

Experimental Procedures

Materials. Human erythrocyte glucose transporter was isolated from donors of blood group A as previously described (Kasahara & Hinkle, 1977; Sase et al., 1982). NaB^3H_4 (341 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 (98%) was obtained from Merck Co., Darmstadt, FRG. β-*N*-Acetylhexosaminidase and α-mannosidase were purified from jack bean meal according to the method of Li and Li (1972). β-Galactosidase and another β-*N*-acetylhexosaminidase were also purified from the culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow et al. (1977). *Aspergillus saitoi* α-mannosidase I was purified according to the method of Amano and Kobata (1986). *Charonia lampas* α-*N*-acetylgalactosaminidase was purchased from Seikagaku Kogyo Co., Tokyo. *Ampullaria* β-mannosidase and *Corynebacterium* sp. α-fucosidase were kindly supplied by Tokyo Zouki Chemical Co., Tokyo, and Takara Shuzo Co., Kyoto, respectively. *Arthrobacter ureafaciens* sialidase and $NaBH_4$ were purchased from Nacalai Tesque, Inc., Kyoto. Newcastle disease virus (NDV) sialidase was purified from NDV B1 strain according to the method

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of Paulson et al. (1982). Endo-β-galactosidase was purified from the culture fluid of *Flavobacterium keratolyticus* according to the method of Kitamikado et al. (1982). Another endo-β-galactosidase from *D. pneumoniae* was purified as described in the previous paper (Takasaki & Kobata, 1976). Bovine epididymal α-fucosidase was purchased from Sigma Chemical Co., St. Louis, MO. *Aleuria aurantia* lectin (AAL)–Sepharose was prepared as reported previously (Yamashita et al., 1985).

Oligosaccharides. The following oligosaccharides were used as standards, and their structural assignments were performed as described in the cited references. Siaα2→6Galβ1→4GlcNAcβ1→2Manα1→6 (Siaα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Sia_2·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$) and Siaα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Sia·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$) were prepared from Bence Jones protein Nei λ (Ohkura et al., 1985). Manα1→2Manα1→6(Manα1→2Manα1→3)Manα1→6(Manα1→2Manα1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man_9·GlcNAc·GlcNAc$_{OT}$) was prepared from the egg jelly coat glycoproteins of starfish by hydrazinolysis followed by reduction with NaB^3H_4 (Endo et al., 1987). Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man_5·GlcNAc·GlcNAc$_{OT}$), GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2 Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·GlcNAc$_{OT}$), GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$), GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ ($GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$), and GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ ($GlcNAc_2$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from immunoglobulin M purified from a patient with Waldenström macroglobulinemia (Ohbayashi et al., 1989). GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (GlcNAc·Man_2·GlcNAc·Man·GlcNAc·GlcNAc$_{OT}$) were obtained from $GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$ and $GlcNAc_2$·Man_2·GlcNAc·Man·GlcNAc·GlcNAc$_{OT}$ by diplococcal β-*N*-acetylhexosaminidase digestion. 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 $GlcNAc_2$·Man_3·GlcNAc·GlcNAc$_{OT}$ and $GlcNAc_2$·Man_3·GlcNAc·Fuc·GlcNAc$_{OT}$ by jack bean β-*N*-acetylhexosaminidase digestion. GalNAcβ1→4(Siaα2→3)Galβ1→4Glc$_{OT}$ (GalNAc·Sia·Gal·Glc$_{OT}$) was obtained from ganglioside (GM2) by endoglycoceramidase digestion (Ito & Yamagata, 1986) followed by reduction with NaB^3H_4.

Analytical Methods. Anion-exchange column chromatography was carried out according to the published procedure (Katoh et al., 1990). Methylation analysis of oligosaccharides was performed as reported in the previous paper (Ohbayashi et al., 1989), using a JEOL DX-300 gas chromatography–mass spectrometer (JEOL Co., Tokyo) equipped with a DB5-30N capillary column (0.25 mm × 30 m, J & W Scientific, Inc., Cordova, CA). The column temperature was programmed from 95 to 280 °C at a rate of 4 °C/min.

Bio-Gel P-4 (under 400 mesh) column chromatography was performed as reported by Yamashita et al. (1982). Identification of sialic acid released from glucose transporter by sialidase digestion was performed by paper chromatography using 1-butanol/acetic acid/water (12:3:5 v/v) after NaB^3H_4 reduction (Endo et al., 1988).

Liberation of the Asparagine-Linked Sugar Chain of Glucose Transporter from Human Erythrocytes. Thoroughly dried glucose transporter (30 mg) was subjected to 9 h of hydrazinolysis followed by N-acetylation as previously described (Takasaki et al., 1982). One-fifth of the oligosaccharide fraction thus obtained was reduced with NaB^3H_4 (400 μCi) in 100 μL of 0.05 N NaOH at 30 °C for 4 h, and the resulting radioactive oligosaccharide was purified as described already (Takasaki et al., 1982).

For structural study of poly-*N*-acetyllactosamine outer chains, another one-fifth of the oligosaccharide fraction was reduced with $NaBH_4$ (1 mg) in the same manner as in the case of NaB^3H_4 reduction.

For methylation analysis, the remaining oligosaccharide fraction was reduced with NaB^2H_4 (2 mg). To facilitate detection of the oligosaccharides, one-tenth of the tritium-labeled oligosaccharide fraction was added to the deuterium-labeled sample.

In order to determine the number of asparagine-linked sugar chains in one molecule of glucose transporter, 10 nmol of lactose was added as an internal standard to the oligosaccharide fraction just before the reduction with NaB^3H_4. [^{3}H]Lactitol and [^{3}H]oligosaccharide fractions were separated by paper chromatography using ethyl acetate/pyridine/acetic acid/water (5:5:1:3 v/v) as a solvent. On the basis of the radioactivities incorporated into lactitol and the oligosaccharide mixture and the molecular weight of the glucose transporter (64 000), it was estimated that the glycoprotein contains approximately one asparagine-linked sugar chain per molecule.

Glycosidase Digestion. Radioactive oligosaccharides [(5–500) × 10^3 cpm] were incubated with one of the following reaction mixtures at 37 °C for 18 h, unless otherwise noted: *A. ureafaciens sialidase digestion*, 50 milliunits of enzyme in 0.1 M sodium acetate buffer, pH 5.0 (50 μL); *NDV sialidase digestion*, 450 milliunits of enzyme in 10 mM sodium cacodylate buffer, pH 6.5 (40 μL) for 2 h; *diplococcal β-galactosidase digestion*, 5 milliunits of enzyme in 0.2 M citrate–phosphate buffer, pH 6.0 (50 μL); *diplococcal β-N-acetylhexosaminidase digestion*, 4 milliunits of enzyme in 0.1 M sodium citrate buffer, pH 6.0 (50 μL); *jack bean β-N-acetylhexosaminidase digestion*, 4 units of enzyme in 0.2 M sodium citrate buffer, pH 5.0 (50 μL); *jack bean α-mannosidase digestion*, 0.8 unit of enzyme in 0.05 M sodium acetate buffer, pH 4.5, containing 1 mM $ZnCl_2$ (50 μL); *A. saitoi α-mannosidase I digestion*, enzyme (50 ng) in 0.1 M sodium acetate buffer, pH 5.0 (40 μL); *Ampullaria β-mannosidase digestion*, 10 milliunits of enzyme in 0.05 M sodium citrate buffer, pH 4.0 (50 μL); *bovine epididymal α-fucosidase digestion*, 10 milliunits of enzyme in 0.05 M sodium citrate buffer, pH 6.0 (40 μL); *Corynebacterium α-fucosidase digestion*, 1 unit of enzyme in 0.1 M sodium phosphate buffer, pH 8.0 (30 μL); *C. lampas α-N-acetylgalactosaminidase digestion*, 0.1 unit of enzyme in 0.1 M sodium citrate buffer, pH 4.0 (50 μL); *digestion with a mixture of diplococcal β-galactosidase and jack bean β-N-acetylhexosaminidase*, 5 milliunits of β-galactosidase and 4 units of β-*N*-acetylhexosaminidase in 0.1 M sodium citrate buffer, pH 5.5 (50 μL);

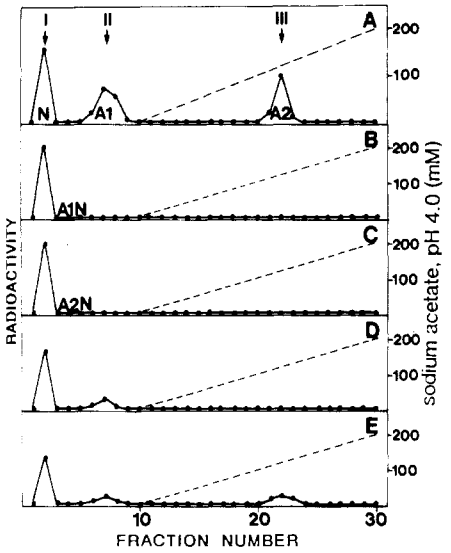

FIGURE 1: Anion-exchange column chromatography of the radioactive oligosaccharides released from glucose transporter by hydrazinolysis. The experimental details are described in the text. Arrows indicate the positions where authentic oligosaccharides eluted: I, Man_3·GlcNAc·$GlcNAc_{OT}$; II, Sia·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$; III, Sia_2·Gal_2·$GlcNAc_2$·Man_3·GlcNAc·$GlcNAc_{OT}$. (A) Radioactive oligosaccharides liberated from glucose transporter of human erythrocytes; (B) radioactive component A1 in (A) digested with *A. ureafaciens* sialidase; (C) radioactive component A2 in (A) digested with *A. ureafaciens* sialidase; (D) radioactive component A1 in (A) digested with NDV sialidase; (E) radioactive component A2 in (A) digested with NDV sialidase.

F. keratolyticus endo-β-galactosidase digestion, 9.3 microunits of enzyme in 0.1 M sodium acetate buffer, pH 6.0, containing 0.1 mg of galactonolactone (50 μL) for 40 h; *diplococcal endo-β-galactosidase digestion*, 0.2 milliunit of enzyme in 0.15 M citrate-phosphate buffer, pH 6.0 (50 μL) for 24 h. A small amount of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Reactions were terminated by heating the reaction mixture in boiling water for 3 min, and then the products were analyzed by Bio-Gel P-4 column chromatography.

Results

Fractionation of Oligosaccharides by Anion-Exchange Column Chromatography. The tritium-labeled oligosaccharide mixture, obtained from glucose transporter by hydrazinolysis, was separated into a neutral (N) and two acidic (A1 and A2) fractions by anion-exchange column chromatography with use of a Mono-Q HR5/5 column (Figure 1A). The molar ratio of oligosaccharides in fractions N, A1, and A2, calculated on the basis of their radioactivities, was 19:16:15. When the acidic fractions (A1 and A2) were incubated with *A. ureafaciens* sialidase, they were completely converted to neutral oligosaccharide mixtures, indicating that only sialic acids are included as the acidic residues in these oligosaccharides (Figure 1B,C). The neutral oligosaccharide fractions obtained from A1 and A2 were named A1N and A2N, respectively. Sialidase of NDV is very useful in determining the sialic acid linkages because it cleaves the Siaα2→3Gal linkage but not the Siaα2→6Gal linkage (Paulson et al., 1982). When fraction A1 was incubated with NDV sialidase, 88% was converted to a neutral component (Figure 1D). In the case of fraction A2, 12% remained unchanged and 9% was converted to monosialyl oligosaccharides (Figure 1E). The NDV sialidase digestion should be complete because the same elution patterns as in Figure 1D,E were obtained after the second NDV sialidase treatment of fractions

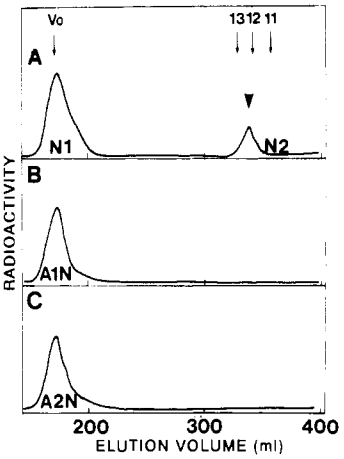

FIGURE 2: Bio-Gel P-4 column chromatography of radioactive oligosaccharide fractions. Arrows indicate the void volume (V_0) and the elution positions of glucose oligomers added as internal standards (the numbers indicate the glucose units). The arrowhead indicates the elution position of authentic Man_9·GlcNAc·$GlcNAc_{OT}$. (A) Radioactive components in fraction N; (B) radioactive components in fraction A1N; (C) radioactive components in fraction A2N.

A1 and A2, respectively (data not shown). These results indicated that fractions A1 and A2 contain 1 and 2 mol of sialic acid residues per molecule, respectively, and that the acidic oligosaccharides of glucose transporter contain both the Siaα2→3Gal and the Siaα2→6Gal linkages. This assumption was further confirmed by methylation analysis which will be described later. The sialic acid residues released from the intact glucose transporter by *A. ureafaciens* sialidase digestion were all *N*-acetylneuraminic acid, and no *N*-glycolylneuraminic acid was detected (data not shown).

Fractionation of the Neutral Oligosaccharides by Bio-Gel P-4 Column Chromatography. Fractions N, A1N, and A2N were subjected to Bio-Gel P-4 column chromatography. As shown in Figure 2A, fraction N was separated into two radioactive peaks (N1 and N2) in a molar ratio of 17:3. Fraction N1 was almost excluded by Bio-Gel P-4, indicating that fraction N1 contained oligosaccharides with molecular weight of larger than 4000. All oligosaccharides in fractions A1N and A2N were eluted at the same position as fraction N1 upon Bio-Gel P-4 column chromatography (Figure 2B,C). When fractions N1, A1N, and A2N were subjected to Bio-Gel P-10 column chromatography, broad peaks which eluted close to the void volume were obtained (data not shown). These results indicated that oligosaccharides in fractions N1, A1N, and A2N were heterogeneous.

Structural Study of Oligosaccharide in Fraction N2. In order to determine the anomeric configuration and sequence of each monosaccharide of oligosaccharide in fraction N2, radioactive fraction N2 was subjected to sequential exoglycosidase digestion and analyzed by Bio-Gel P-4 column chromatography. By incubation with *A. saitoi* α-mannosidase I, which specifically cleaves the Manα1→2Man linkage (Yamashita et al., 1980), N2 was converted to a radioactive component with the same mobility as authentic Man_5·GlcNAc·$GlcNAc_{OT}$ with release of 4 mol of mannose residues (Figure 3A). The radioactive product in Figure 3A was then converted to a radioactive component with the same elution position as authentic Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$ by incubation with jack bean α-mannosidase (data not shown). The structure of the radioactive trisaccharide was confirmed by sequential digestion with *Ampullaria* β-mannosidase and jack bean β-*N*-acetylhexosaminidase. Methylation analysis of oligosaccharide in fraction N2 gave the results shown in

Table I: Methylation Analysis of Fractions N1, N2, A1N plus A2N, and A1 plus A2 and the Core Portion of N1

methylated sugar	molar ratio[a]				
	N1	N1-1[b]	N2	A1N plus A2N	A1 plus A2
fucitol					
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.4	0.4		0.3	0.3
galactitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	3.4			3.1	1.4
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)					0.2
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	12.5			12.6	14.1
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.4			1.5	1.5
4,6-di-*O*-methyl (1,2,3,5-tetra-*O*-acetyl)	tr[c]				
mannitol					
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)			2.8		
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	*2.0*	2.1	3.7	*2.0*	*2.0*
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	0.5	0.5	1.9	0.3	0.3
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	tr	tr		tr	tr
2-mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	0.5	0.5		0.6	0.6
2-(*N*-methylacetamido)-2-deoxyglucitol					
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.5	2.4		0.5	0.5
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	17.7	*1.0*	*1.0*	17.8	17.8
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.6	0.6		0.5	0.5
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.3	0.3	0.8	0.4	0.4
2-(*N*-methylacetamido)-2-deoxygalactitol					
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	tr				

[a] Numbers in the table were calculated by taking the italic values as integers. [b] After *F. keratolyticus* endo-β-galactosidase digestion. [c] Less than 0.1.

Table I. These results indicated that the structure of oligosaccharide in fraction N2 should be Man_9·GlcNAc·$GlcNAc_{OT}$ as shown in Figure 7.

Structures of the Core Portion of Oligosaccharides in Fractions N1, A1N, and A2N. When subjected to AAL–Sepharose column chromatography (Yamashita et al., 1985), all three neutral oligosaccharide fractions (N1, A1N, and A2N) were separated into two groups. The fractions retained by an AAL–Sepharose column and eluted with 1 mM L-fucose were named as N1(+F), A1N(+F), and A2N(+F) and those not retained as N1(−F), A1N(−F), and A2N(−F), respectively. Taking the binding specificity of the AAL–Sepharose column into account (Yamashita et al., 1985), all oligosaccharides in fractions N1(+F), A1N(+F), and A2N(+F) were assumed to contain the fucose residue linked at the C-6 position of the proximal *N*-acetylglucosamine residue of their trimannosyl core, and those in fractions N1(−F), A1N(−F), and A2N(−F) lack the fucose residue. This estimation was supported by the results of methylation analysis and exo- and endoglycosidase digestion as will be described later.

When the radioactive oligosaccharides in fraction N1(−F) were incubated with a mixture of diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase, most of them were converted to a radioactive oligosaccharide with the same mobility as authentic Man_3·GlcNAc·$GlcNAc_{OT}$ (Figure 3B, dashed line). The structure of this component was confirmed to be Manα1→(Manα1→)Manβ1→GlcNAcβ1→$GlcNAc_{OT}$ by sequential digestion with jack bean α-mannosidase, *Ampullaria* β-mannosidase, and jack bean β-*N*-acetylhexosaminidase (data not shown). On the other hand, those in fraction N1(+F) were converted to a radioactive oligosaccharide with the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ by the same treatment (Figure 3B, solid line). The structure of this component was confirmed to be Manα1→(Manα1→)Manβ1→GlcNAcβ1→(Fucα1→)$GlcNAc_{OT}$ by sequential digestion with jack bean α-mannosidase, *Ampullaria* β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and epididymal α-fucosidase (data not shown). These results together with the detection of 1,3,5,6-tetra- and 1,3,5-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitols by methylation analysis (Table I) indicated that fraction N1 was concluded to be a mixture of the complex-type oligosaccharides with nonfucosylated and fucosylated trimannosyl cores. On the basis of the substrate specificity of diplococcal β-galactosidase, which cleaves the Galβ1→4GlcNAc linkage but not the Galβ1→3GlcNAc and the Galβ1→6GlcNAc linkages (Paulson et al., 1978), it was concluded that the Galβ1→GlcNAc groups in their outer-chain moieties occur exclusively as Galβ1→4GlcNAc. De-

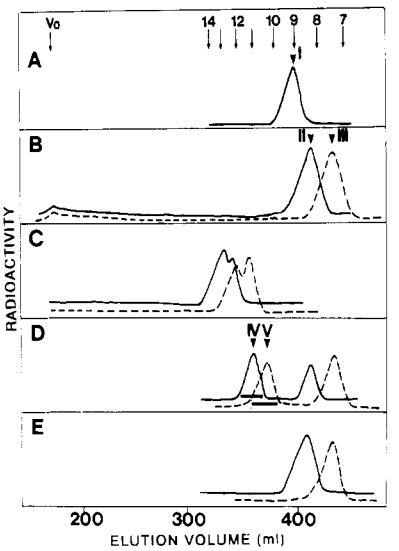

FIGURE 3: Glycosidase digestion of the radioactive oligosaccharides in fractions N2, N1(−F), and N1(+F). The arrows at the top of the figure are the same as in Figure 2. The arrowheads indicate the elution positions of authentic oligosaccharides: I, Man_5·GlcNAc·$GlcNAc_{OT}$; II, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; III, Man_3·GlcNAc·$GlcNAc_{OT}$; IV, GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$; V, GlcNAc·Man_2·GlcNAc·Man·GlcNAc·$GlcNAc_{OT}$. (A) Fraction N2 in Figure 2A after incubation with *A. saitoi* α-mannosidase I; (B) fractions N1(−F) (dashed line) and N1(+F) (solid line) after incubation with a mixture of diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase; (C) fractions N1(−F) (dashed line) and N1(+F) (solid line) after incubation with *F. keratolyticus* endo-β-galactosidase; (D) *dashed line* and *solid line* represent the products obtained from the *dashed-line* and *solid-line* components in (C) by incubation with diplococcal β-*N*-acetylhexosaminidase, respectively; (E) *dashed line* and *solid line* represent the products obtained from the *dashed-line* and *solid-line* components indicated by bars in (D) by incubation with jack bean β-*N*-acetylhexosaminidase, respectively.

Table II: Proposed Core Structures for the Oligosaccharides Released from Glucose Transporter of Human Erythrocytes by Hydrazinolysis

structures	fractions[a] N1	A1N	A2N
Manα1→6 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	22.6	20.1	20.3
Fucα1 ↓ Manα1→6 6 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	26.1	12.0	12.6
GlcNAcβ1 ↓ Manα1→6 4 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	17.4	30.9	30.7
GlcNAcβ1 Fucα1 ↓ ↓ Manα1→6 4 6 Manβ1→4GlcNAcβ1→4GlcNAc Manα1→3	33.9	37.0	36.4

[a] Numbers indicate the percent molar ratio in each fraction.

tection of only 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol as the di-*O*-methyl derivative of *N*-acetylglucosamine by methylation analysis of N1 (Table I) also supported this conclusion.

When digested with *F. keratolyticus* endo-β-galactosidase, fraction N1(−F) and fraction N1(+F) were converted to a mixture of radioactive components as shown by the dashed line and the solid line in Figure 3C, respectively. Upon incubation with diplococcal β-*N*-acetylhexosaminidase, the dashed-line components in Figure 3C were converted to a mixture of radioactive components with the same mobilities as authentic GlcNAc·Man_2·GlcNAc·Man·GlcNAc·$GlcNAc_{OT}$ and Man_3·GlcNAc·$GlcNAc_{OT}$ (Figure 3D, dashed line). On the other hand, the solid-line components in Figure 3C were converted a mixture of radioactive components with the same mobilities as authentic GlcNAc·Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$ and Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 3D, solid line). By jack bean β-*N*-acetylhexosaminidase digestion, the dashed line peak with mobility of 10.4 glucose units in Figure 3D was converted to the nonfucosylated trimannosyl core, releasing two *N*-acetylglucosamine residues (Figure 3E, dashed line). The radioactive solid-line component with mobility of 11.0 glucose units was converted to the fucosylated trimannosyl core, releasing two *N*-acetylglucosamine residues by the same treatment (Figure 3E, solid line). As reported previously (Yamashita et al., 1981), diplococcal β-*N*-acetylhexosaminidase cleaves the GlcNAcβ1→2Man linkage but not that on the Manα1→6 arm of the bisected biantennary complex-type oligosaccharide while jack bean β-*N*-acetylhexosaminidase can cleave all GlcNAcβ1→ linkages. These results together with the detection of 3,4,6-tri-, 2,4-di-, and 2-mono-*O*-methylmannitols and 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol by methylation analysis of N1-1 (Table I) indicated that the structures of the radioactive products by endo-β-galactosidase digestion were

±GlcNAcβ1 ±Fucα1
↓ ↓
GlcNAcβ1→2Manα1→6 4 6
Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$
GlcNAcβ1→2Manα1→3

The core structures of A1N and A2N were determined in the same way, and the percent molar ratios of each core structure

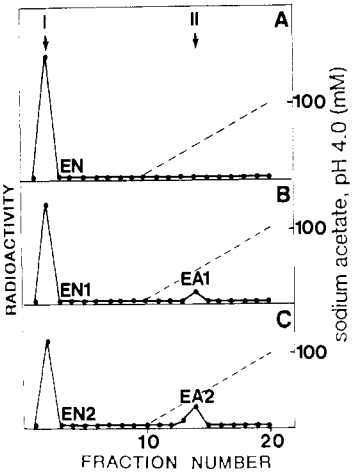

FIGURE 4: Anion-exchange chromatography of outer-chain fragments released by *F. keratolyticus* endo-β-galactosidase digestion. Arrows indicate the positions where authentic oligosaccharides eluted: I, lactitol; II, GalNAc·Sia·Gal·Glc_{OT}. (A) Radioactive fragments obtained from fraction N; (B) radioactive fragments obtained from fraction A1; (C) radioactive fragments obtained from fraction A2.

were calculated on the basis of the radioactivities and are summarized in Table II.

Detection of a trace amount of 3,6-di-*O*-methylmannitol by methylation analysis of N1 (Table I) indicated that 2,4-branched triantennary oligosaccharide might be included in this fraction as a very minor component. The results so far described indicated that the complex-type sugar chains of glucose transporter are mostly the biantennary forms containing the poly-*N*-acetyllactosamine outer chains.

Structures of Oligosaccharides Released by Endo-β-galactosidase Digestion. In order to determine the structures of the outer-chain moieties, the following studies were performed. First, the oligosaccharide mixture reduced with $NaBH_4$ (see Experimental Procedures) was subjected to anion-exchange column chromatography as described above. Components corresponding to fractions N, A1, and A2 in Figure 1A were collected, desalted by passing through a small column of Dowex 50W-X12 (H^+ form) (0.5 × 3 cm), and then evaporated to dryness. The samples were digested with *F. keratolyticus* endo-β-galactosidase and then reduced with NaB^3H_4. The radioactive products were analyzed by chromatography with use of a Mono-Q HR5/5 column. Although fraction N gave only a neutral component (EN in Figure 4A), fractions A1 and A2 gave one neutral and one acidic component as shown in Figure 4, panels B and C, respectively. The molar ratio of oligosaccharides in fractions EN1 and EA1 and that in fractions EN2 and EA2, calculated on the basis of their radioactivities, were 13:1, and 6:1, respectively. Since fraction EA1 and fraction EA2 gave the same results in a series of structural analyses, only the data for fraction EA2 will be documented below.

That oligosaccharide in fraction EA2 was monosialylated was confirmed by *A. ureafaciens* sialidase digestion and by mild acid treatment followed by anion-exchange column chromatography in the same manner as described already (data not shown). The neutral oligosaccharide fraction obtained from EA2 was named EA2N. When fraction EA2N was subjected to Bio-Gel P-4 column chromatography, it was eluted at 4.4 glucose units (Figure 5A). The radioactive component in Figure 5A released one galactose residue by incubation with diplococcal β-galactosidase (Figure 5B) and then converted to radioactive galactitol with release of one *N*-acetylglucosamine residue by incubation with jack bean β-*N*-acetylhexosaminidase (Figure 5C). These results indi-

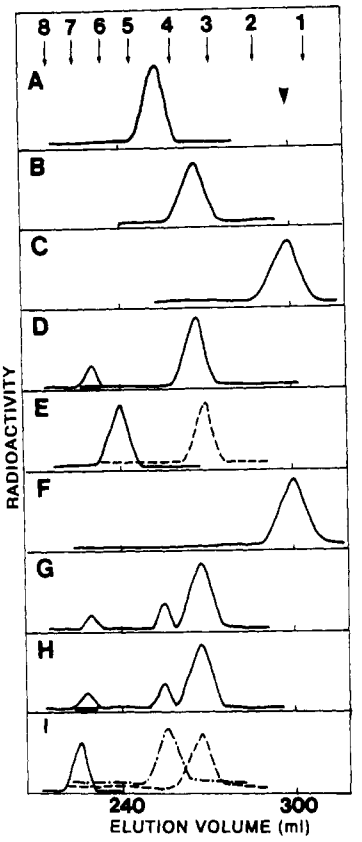

FIGURE 5: Bio-Gel P-4 column chromatography of radioactive oligosaccharides released by *F. keratolyticus* endo-β-galactosidase digestion and their sequential exoglycosidase digestion products. The arrows are the same as in Figure 2. The arrowhead indicates the elution position of galactitol. (A) Radioactive component in fraction EA2N; (B) radioactive peak in (A) after incubation with diplococcal β-galactosidase; (C) radioactive peak in (B) after incubation with jack bean β-*N*-acetylhexosaminidase; (D) radioactive components in fraction EN2; (E) radioactive peak indicated by a bar in (D) after incubation with diplococcal β-galactosidase (solid line) or with sequential jack bean β-*N*-acetylhexosaminidase and diplococcal β-galactosidase (dashed line); (F) solid-line component in (E) after incubation with jack bean β-*N*-acetylhexosaminidase; (G) radioactive components in fraction EN1; (H) radioactive components in fraction EN; (I) remaining component in the radioactive peak indicated by a bar in (H) after incubation with diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase (solid line), and after digestion either by sequential *C. lampas* α-*N*-acetylgalactosaminidase and *Corynebacterium* α-fucosidase (dot-dash line) or by diplococcal endo-β-galactosidase (dashed line).

cated that the structure of oligosaccharide in fraction EA2N (Figure 5A) should be Galβ1→4GlcNAcβ1→3Gal_{OT} and that in fraction EA2 (and also in fraction EA1) is Neu5Acα2→3(6)Galβ1→4GlcNAcβ1→3Gal_{OT}.

When fraction EN2 was subjected to a Bio-Gel P-4 column, it gave two components eluting at 6.4 and 3.6 glucose units in a molar ratio of 1:7 (Figure 5D). Since the radioactive component eluted at 3.6 glucose units gave the same analytical data as the radioactive component in Figure 5B, its structure should be GlcNAcβ1→3Gal_{OT}. By digestion with diplococcal β-galactosidase, the radioactive component at 6.4 glucose units was converted to an oligosaccharide with the effective size of 5.5 glucose units, releasing one galactose residue (Figure 5E, solid line). Upon digestion with jack bean β-*N*-acetylhexosaminidase, the radioactive solid-line component in Figure 5E was converted to galactitol, releasing two *N*-acetylglucosamine residues (Figure 5F). These results indicated that the structure of the component eluted at 6.4 glucose units in Figure 5D is Galβ1→4GlcNAcβ1→6(and/or 3)[GlcNAcβ1→3(and/or 6)]Gal_{OT}. Detection of 2,4-di-*O*-methylgalactitol by methylation analysis of N1 supported this conclusion (Table I). However, the location of galactose residue could not be determined by the data obtained so far. In order to determine the location of the Galβ1→4GlcNAcβ1→ group, the radioactive component at 6.4 glucose units was subjected to another series of sequential exoglycosidase digestions. When it was digested sequentially with jack bean β-*N*-acetylhexosaminidase and then with diplococcal β-galactosidase, it was converted to a component eluted at 3.4 glucose units with the release of one *N*-acetylglucosamine and one galactose residue (Figure 5E, dashed line). By digestion with jack bean β-*N*-acetylhexosaminidase, this radioactive component was converted to galactitol, releasing an *N*-acetylglucosamine residue (data not shown). This result indicated that the radioactive component eluted at 3.4 glucose units was GlcNAcβ1→6Gal_{OT} and/or GlcNAcβ1→3Gal_{OT}. In order to discriminate between these disaccharide isomers, the radioactive component eluted at 3.4 glucose units was subjected to the column of immobilized *Psathyrella velutina* lectin which was specific for *N*-acetylglucosamine (Kochibe & Matta, 1989). It was confirmed that GlcNAcβ1→6Gal_{OT} was only retarded and GlcNAcβ1→3Gal_{OT} bound to the column (Endo et al., manuscript in preparation). All of the radioactive disaccharide in Figure 5E (dashed line) was retarded in the column (data not shown), indicating that the structure of the component eluted at 6.4 glucose units in Figure 5D is Galβ1→4GlcNAcβ1→6-(GlcNAcβ1→3)Gal_{OT}. This tetrasaccharide was never sialylated because it could not be detected in fraction EA2N (Figure 5A). These results indicated that fraction EN2 should be derived from inner parts of poly-*N*-acetyllactosamine outer chains, and fraction EA2 was from the peripheral moiety of each outer chain (Figure 6). The molar ratio of three oligosaccharide fractions (the peak in Figure 5A and the two peaks in Figure 5D) calculated from their radioactivities was 4:3:21. Because the integer of sialylated oligosaccharide in the fraction EA2 should be two, it could be estimated that the outer chains of oligosaccharides in fraction A2 are constructed by the following components: [Neu5Acα2→3(6)Galβ1→4GlcNAcβ1→3Galβ1→4]$_2$, [Galβ1→4[GlcNAcβ1→6(3)]-Galβ1→4]$_{1\sim2}$, (GlcNAcβ1→3Galβ1→4)$_{10\sim11}$, and (GlcNAcβ1→2Manα1→)$_2$.

In the case of fraction EN1, three components were obtained by Bio-Gel P-4 column chromatography as shown in Figure 5G. Sequential exoglycosidase digestion of the component eluted at 4.4 glucose units revealed that it was identical with fraction EA2N (Figure 5A). Two other components were identical with the two radioactive components in Figure 5D. Since fraction A1 was a mixture of monosialylated oligosaccharides, the component eluted at 4.4 glucose units in Figure 5G, Galβ1→4GlcNAcβ1→3Gal_{OT}, should be derived from the nonsialylated terminal portion of the poly-*N*-acetyllactosamine outer chain.

Fraction EN also gave three radioactive peaks as shown in Figure 5H. Sequential exoglycosidase digestion of the components eluted at 4.4 and 3.5 glucose units gave the same results as the corresponding components in Figure 5G. Approximately 90% of the component in the peak at around 6.4 glucose units gave the same results as the corresponding component in Figure 5D, indicating that it is Galβ1→4GlcNAcβ1→6(GlcNAcβ1→3)Gal_{OT}. The remaining 10% of this peak at 7.0 glucose units (Figure 5I, solid line) was susceptible to neither diplococcal β-galactosidase digestion nor jack bean β-*N*-acetylhexosaminidase digestion (Figure 5I, solid line). However, it was converted to Galβ1→4GlcNAcβ1→3Gal_{OT} by sequential digestion with *C. lampas* α-*N*-acetyl-

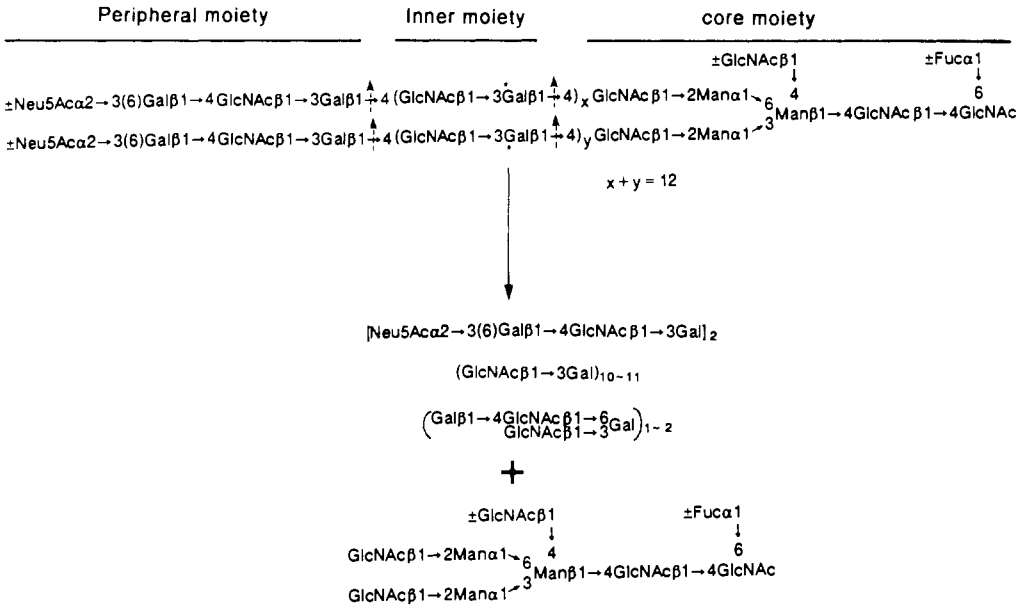

FIGURE 6: Composite diagram of the oligosaccharides in fraction A2 (Figure 1) obtained from human erythrocyte glucose transporter. The broken up arrows indicate the cleavage sites by *F. keratolyticus* endo-β-galactosidase digestion. The molar ratio of each fragment released by endo-β-galactosidase treatment was calculated from radioactivity incorporated after NaB^3H_4 reduction and fixed the sialylated tetrasaccharides as two. See details in the text. (*) One or two galactose residues are substituted by the Galβ1→4GlcNAcβ1→6 group.

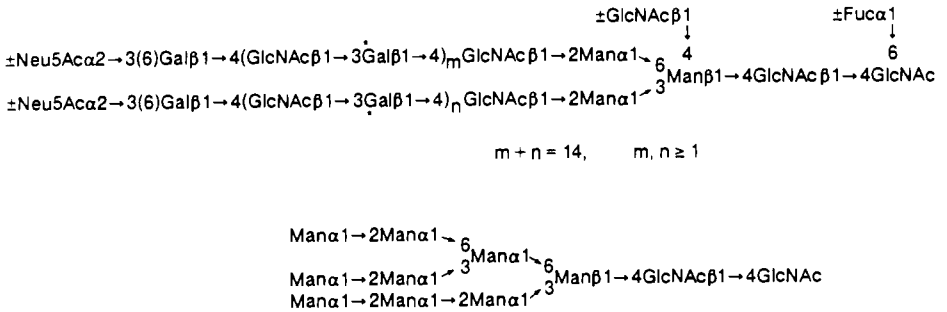

FIGURE 7: Proposed structures of the asparagine-linked sugar chains of glucose transporter purified from human erythrocytes. (*) One or two galactose residues are substituted by the Galβ1→4GlcNAcβ1→6 group. Part of the oligosaccharides in fraction N contain blood group A determinant.

galactosaminidase and *Corynebacterium* α-fucosidase (Fig. 5I, dot-dash line) and to GlcNAcβ1→3Gal_{OT} by digestion with diplococcal endo-β-galactosidase which cleaves trisaccharides only from blood group determinants A and B composed of type 2 chain (Takasaki & Kobata, 1976) (Figure 5I, dashed line). These results, and the detection of trace amounts of 4,6-di-*O*-methylgalactitol and 3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxygalactitol by methylation analysis of N1 (Table I), indicated that a part of oligosaccharides in fraction N1 may include the blood group A determinant GalNAcα1→3(Fucα1→2)Galβ1→ as a peripheral structure.

Based on the studies described above, structures of oligosaccharide of glucose transporter were proposed as shown in Figure 7.

Discussion

Both high-mannose-type and biantennary complex-type sugar chains are found in the glucose transporter. Many glycoproteins containing high-mannose-type sugar chains have been studied. Generally, these structures occur as a series of high-mannose-type forms (Hubbard & Ivatt, 1981; Kornfeld & Kornfeld, 1985; Ohbayashi et al., 1989). Therefore, the occurrence of only Man_9·GlcNAc·GlcNAc as the high-mannose-type sugar chains in the glucose transporter is of particular interest. This evidence indicates that a part of the Man_9·GlcNAc·GlcNAc is not processed by the α-mannosidase in endoplasmic reticulum while the remainder are completely processed to complex-type sugar chains. A similar phenomenon was found in the sugar chain of β-glucuronidase purified from rat preputial gland. This glycoprotein contains Man_9·GlcNAc·GlcNAc and Man_6·GlcNAc·GlcNAc in a 2:3 molar ratio (T. Mizuochi, K. Kato, and A. Kobata, unpublished result). The enzymatic basis of these interesting phenomena remains to be elucidated.

The complex-type sugar chains of glucose transporter contain four variants as their core portions, Manα1→6(±GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc, and poly-*N*-acetyllactosamine groups as their outer-chain moieties. While the length of poly-*N*-acetyllactosamine groups was a heterogeneous distribution as documented by the gel permeation study with Bio-Gel P-10, their average size was 16 *N*-acetyllactosamine units. This structural evidence is consistent with the previous finding that the glucose transporter of human erythrocyte is susceptible to endo-β-galactosidase digestion and is heterogeneously glycosylated (Gorga et al., 1979).

A comment should be added to the sialyl linkage of poly-*N*-acetyllactosamine outer-chain moieties. Although glycophorin A has the Neu5Acα2→6Gal linkage only (Yoshima et al., 1980a), the glucose transporter has preferentially the Neu5Acα2→3Gal linkage as shown in this study. It is consistent with the findings that the longer poly-*N*-acetyllactosamine chain of band 3 has the Neu5Acα2→3Gal linkage whereas the shorter chain has the Neu5Acα2→6Gal linkage

(Fukuda et al., 1984b). Possibly, the longer *N*-acetyllactosamine chain has much higher affinity for α2→3sialyltransferase than α2→6sialyltransferase of human erythroblasts.

Structural features of the sugar moieties of two major intrinsic glycoproteins of human erythrocytes, glycophorin A and band 3, have been studied in detail (Yoshima et al., 1980a; Tsuji et al., 1980, 1981; Fukuda et al., 1984b). Yoshima et al. (1980a) reported that glycophorin A has a biantennary complex-type sugar chains with the Neu5Acα2→6Galβ1→4GlcNAc group as its outer chain. The special features of the sugar chains are that they include a bisecting *N*-acetylglucosamine residue and the α-fucosyl residue linked at the C-6 position of the proximal *N*-acetylglucosamine residue. On the other hand, band 3 was found to have both low and high molecular weight oligosaccharides (Tsuji et al., 1980, 1981; Fukuda et al., 1984b). The low molecular weight oligosaccharides are of biantennary complex type similar to those found in glycophorin A, but part of them lack galactose, fucose, and/or bisecting *N*-acetylglucosamine residues. The high molecular weight ones are also of biantennary complex type but contain extremely heterogeneous poly-*N*-acetyllactosamine groups in their outer-chain moieties. It was also revealed that the groups contain branch structures [→6(3)Galβ1→] as well as probably blood group A, B, and H determinants.

The common structural feature revealed by the studies of the sugar chains of individual glycoproteins of human erythrocytes membrane is that no more highly branched complex-type sugar chains than biantennary are included in these glycoproteins (Yoshima et al., 1980a; Tsuji et al., 1980, 1981; Fukuda et al., 1984b). Although the sugar chains of surface glycoproteins from many types of animal cells have been studied (Yoshima et al., 1980b; Spooncer et al., 1984; Fukuda et al., 1984c; Mizoguchi et al., 1984; Yamashita et al., 1984; Dennis et al., 1986; Santer et al., 1989; Hara et al., 1989), only human erythrocytes show such a unique characteristic. Therefore, it is possible that the expression of the *N*-acetylglucosaminyltransferase IV, which makes the GlcNAcβ1→4Manα1→3 group, and of the *N*-acetylglucosaminyltransferase V, which is responsible for the formation of the GlcNAcβ1→6Manα1→6 group, might be suppressed in human erythrocytes. However, a study of the surface sugar chains of erythroid leukemic K562 cell has revealed that it has tri- and tetraantennary complex-type sugar chains together with a biantennary one (Yoshima et al., 1982). Furthermore, only a small portion of the sugar chains contains poly-*N*-acetyllactosamine outer chains (Turco et al., 1980; Yoshima et al., 1982). These results indicated that the sugar pattern of human erythrocytes changes during their developmental stage, although the possibility that a part of the altered glycosylation may be ascribed to malignant transformation of the cells cannot be eliminated (Yamashita et al., 1984; Dennis et al., 1986; Santer et al., 1989). It is also of interest that Fukuda et al. (1984a) reported that the poly-*N*-acetyllactosamine groups of the sugar moiety of band 3 isolated from human umbilical cord erythrocytes were shorter than those from adult human erythrocytes.

The different structural features are also revealed as described below. First, no high-mannose-type sugar chain was found in band 3 and glycophorin A. Second, the biantennary complex-type sugar chains containing poly-*N*-acetyllactosamine group are not detected in glycophorin A at all. On the other hand, the only glucose transporter did not have low molecular weight oligosaccharides. Finally, the amount of branches and blood group determinants in poly-*N*-acetyllactosamine groups is much smaller in glucose transporter than in band 3. It is of particular interest that three glycoproteins carry distinctly different sugar chains. Since all of them are synthesized in the same cell, the lack of poly-*N*-acetyllactosamine group in glycophorin A cannot be ascribed to the lack of glycosyltransferases responsible for the elongation of outer-chain moieties. One possibility is due to the difference of the molecular organization of glycoproteins. It has been shown that glycophorin A penetrates the lipid bilayer once (Tomita et al., 1978), whereas band 3 and glucose transporter are considered to traverse the lipid bilayer many times (Kopita & Lodish, 1985; Mueckler et al., 1985). Accessibility of glycosyltransferases may be affected by such a difference in molecular arrangement. Another possibility is the protein-specific recognition by some glycosyltransferases. It was reported that lysosomal enzymes contain an unidentified common feature encoded within their peptide, which is recognized by *N*-acetylglucosamine-1-phosphotransferase and results in selective addition of *N*-acetylglucosamine 1-phosphate to the C-6 position of a mannose residue of its high-mannose-type sugar chains (Lang et al., 1984). Another example is that α–β complex of glycohormones may be recognized by an *N*-acetylgalactosaminyltransferase present in the pituitary (Smith & Baenziger, 1988). A third example is the finding that a β1→4galactosyltransferase specific for IgG molecule may be included in human B cells (Furukawa et al., 1990). These data suggested that the processing and maturation of sugar chains of glycoproteins are regulated by complicated mechanisms. It is possible that the elongation enzyme, β1→3-*N*-acetylglucosaminyltransferase, which is responsible for the formation of the GlcNAcβ1→3Gal linkage (Kornfeld & Kornfeld, 1985), is regulated by a specific peptide sequence and/or conformation. Further studies are necessary for understanding the formation of the poly-*N*-acetyllactosamine group and the formation of distinctly different sugar chains in glycoproteins of the same cell.

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