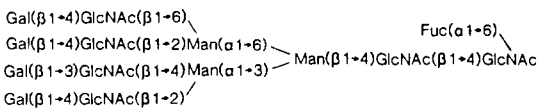
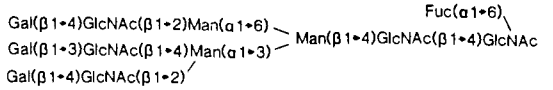
Comparative Structural Study of N-Linked Oligosaccharides of Urinary and Recombinant Erythropoietins†

Eisuke Tsuda, Masaaki Goto, Akihiko Murakami, Kunihisa Akai, Masatsugu Ueda, and Gosei Kawanishi
Research Institute of Life Science, Snow Brand Milk Products Company Ltd., Tochigi 329-05, Japan
Noriko Takahashi*
Department of Biochemistry, Nagoya City University Medical School, Nagoya 467, Japan
Ryuzo Sasaki and Hideo Chiba
Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
Hideko Ishihara, Masami Mori, and Setsuzo Tejima
Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467, Japan
Satoshi Endo and Yoji Arata
Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Tokyo 113, Japan
Received December 3, 1987; Revised Manuscript Received February 25, 1988

ABSTRACT: The structures of the N-linked oligosaccharides of the urinary erythropoietin (u-EPO) purified from urine of aplastic anemic patients were analyzed and compared with those for recombinant erythropoietin (r-EPO) prepared with baby hamster kidney (BHK) cells. Asparagine-linked neutral oligosaccharides were released from each EPO protein by *N*-oligosaccharide glycopeptidase (almond) digestion. The reducing ends of the oligosaccharide chains thus obtained were aminated with a fluorescent reagent, 2-aminopyridine, and the mixture of pyridylamino derivatives of the oligosaccharides was separated by high-performance liquid chromatography (HPLC) on an ODS silica column. More than 8 and 13 kinds of oligosaccharide fractions for u-EPO and r-EPO (BHK), respectively, were completely separated by the one-step HPLC procedure. The structure of each oligosaccharide thus isolated was analyzed by a combination of sequential exoglycosidase digestion and another kind of HPLC with an amide–silica column. Furthermore, high-resolution proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and methylation analyses were carried out in the case of r-EPO (BHK). It has been found that (1) significantly different distributions of oligosaccharide components exist in u-EPO preparations isolated from different individuals, whereas the distribution of oligosaccharide components is quite similar for different r-EPO (BHK) preparations, (2) in r-EPO (BHK) a tetraantennary oligosaccharide with a fucose residue is predominant and various higher molecular weight tetraantennary oligosaccharides with one to three Gal(β1→4)GlcNAc repeating units exist, (3) all of the eight kinds of oligosaccharides of u-EPO also exist in r-EPO (BHK), although the distribution of the oligosaccharides is different for the two proteins, and (4) oligosaccharides with unusual structures containing the Gal(β1→3)GlcNAc group exist in both u- and r-EPO (BHK) (fraction G), and in r-EPO (BHK) (fraction K):



G



K

Erythropoietin regulates the level of mammalian hematopoiesis, and it has been applied as a potential therapeutic reagent for anemia. The urinary erythropoietin (u-EPO)[1] is purified from the urine of patients with aplastic anemia (Miyake et al., 1977; Yanagawa et al., 1984). u-EPO is a sialoglycoprotein with a M_r of 34 000–39 000 (Jacobs et al., 1985) and was confirmed to possess three glycosylation sites (Asn-24, -38, and -83). The composition and the role of the carbohydrate moiety of u-EPO have been reported by Dordal et al. (1985).

Erythropoietin has been prepared by recombinant DNA technique with mammalian cells including baby hamster kidney (BHK) cells (Powell et al., 1986), mouse embryo ψ2 (ψ2) cells, and Chinese hamster ovary (CHO) cells (Egrie et al., 1986). Since the carbohydrate structure can influence the biological properties of the proteins, structural knowledge of the carbohydrate chains of recombinant erythropoietin (r-EPO) is important for clinical use.

Sasaki et al. (1987) have recently reported the detailed structures of both N-linked and O-linked oligosaccharides of r-EPO produced in CHO cells. Especially, they elucidated the number and the localization of *N*-acetylneuraminic acids

† This work was supported in part by the Ministry of Education, Science and Culture of Japan.
* To whom correspondence should be addressed.

[1] Abbreviations: EPO, erythropoietin; r-EPO, recombinant EPO; u-EPO, urinary EPO; BHK, baby hamster kidney; PA, pyridylamino; Fuc, L-fucose, Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance.

0006-2960/88/0427-5646$01.50/0

in side chains. The neutral oligosaccharides reported, however, were only separated as groups according to their molecular sizes. Sasaki et al. analyzed each group of the oligosaccharide by fast atom bombardment–mass spectrometry and methylation analysis and proposed the respective oligosaccharide structures. Complete isolation of all of the individual oligosaccharides in EPO has not yet been accomplished. The analysis of such groups may often result in an erroneous conjecture of the structure of each oligosaccharide contained in the group. To determine precisely the structures, we separated completely neutral oligosaccharides, even those of the same molecular weight, by the one-step HPLC procedure with a reverse-phase column and then directly subjected each oligosaccharide of r-EPO (BHK) to ^{1}H NMR measurement and methylation analysis.

Although our results obtained from r-EPO (BHK) were similar in many respects to those from r-EPO (CHO) reported by Sasaki et al., there were important differences between their report and ours: (1) They maintained that the carbohydrate moiety of u-EPO was indistinguishable from that of r-EPO (CHO). We noticed, however, significant differences between u-EPO and r-EPO (BHK); e.g., the tetraantennary structure with a *N*-acetyllactosaminyl repeat that existed in r-EPO (BHK) was barely detectable in u-EPO. (2) We observed that in both u- and r-EPO (BHK) there existed a significant amount of a tetraantennary oligosaccharide with a Gal(β1→3)GlcNAc sequence (fraction G), whereas Sasaki et al. did not describe the fact. (3) r-EPO (BHK) contained no detectable amount of a triantennary oligosaccharide with a Gal(β1→4)GlcNAc repeating unit that exists in r-EPO (CHO).

Experimental Procedures

Enzymes and Standard Oligosaccharides. *N*-Oligosaccharide glycopeptidase (EC 3.5.1.52) from almond (obtainable as glycopeptidase A), β-galactosidase from *Charonia lampas*, and β-*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo. α-L-Fucosidase from bovine kidney was purchased from Boehringer Mannheim Biochemicals. Pepsin was purchased from Sigma Chemical Co. Standard oligosaccharides described below were prepared by glycopeptidase digestion as described previously (Tomiya et al., 1987). Biantennary oligosaccharides with and without fucose residue were prepared from normal human IgG; tri- and tetraantennary oligosaccharides without fucose residue were prepared from α_1-acid glycoprotein (Yoshima et al., 1981); fucose-containing tri- and tetraantennary oligosaccharides were prepared from porcine pancreatic lipase; Galβ(1,3)GlcNAc-TRI was prepared from fetuin (Townsend et al., 1986). The isomaltooligosaccharides of 4–19 glucose units were prepared from the acid hydrolysate of dextran (Nishigaki et al., 1978).

Other Chemicals. The following materials were purchased from the sources indicated: Sephadex G-15 was from Pharmacia Fine Chemicals; Bio-Gel P-4 (200–400 mesh) was from Bio-Rad; sodium cyanoborohydride was from Aldrich; 2-aminopyridine was from Wako Pure Chemical Industries.

Preparation of u-EPO. Human urinary erythropoietin was isolated from urine of two arbitrary groups of aplastic anemic patients by use of an immunoadsorbent column of monoclonal antibodies and a Sephadex G-100 column as previously described (Yanagawa et al., 1984).

Preparation of r-EPO (BHK). Human EPO protein was produced by a recombinant DNA technique with BHK cells as recipient cells. The plasmid expression vector carrying the human genomic EPO gene was transfected to BHK cells by the calcium phosphate method. The cells were selected for the presence of EPO activity in the supernatant. The selected clonal cells were cultured in basal minimum essential medium containing 1.5% (v/v) new born calf serum for 3 or 4 days. r-EPO (BHK) was purified from the cultured media according to the method used for u-EPO except for the use of an hydroxylapatite column (Goto et al., 1988).

Preparation of Oligosaccharides from Erythropoietin. A total of 300 μg of u-EPO and 20 mg of r-EPO (BHK) was used for the structural analysis of oligosaccharides. Each of erythropoietin samples was desialylated by mild acid hydrolysis at pH 2, 90 °C for 1 h. Desialylated and pepsin-digested erythropoietins were treated with *N*-oligosaccharide glycopeptidase as described by Tomiya et al. (1987). The oligosaccharide fraction was collected by gel filtration on a Bio-Gel P-4 column and further purified by passing it through columns of the ion exchange resins Dowex 50W-X8 (H^+) and Amberlite IRA-400 (CO_3^{2-}) (Nishibe & Takahashi, 1981).

Isolation and Analysis of Pyridylamino Oligosaccharides by HPLC. Asialooligosaccharide fractions obtained as above were reductively aminated with 2-aminopyridine by use of sodium cyanoborohydride (Hase et al., 1984). The pyridylamino derivatives of oligosaccharides thus prepared were purified by gel filtration on a Sephadex G-15 column and were fractionated and identified by HPLC: (1) on a reverse-phase, Shim-pak CLC-ODS column (6 × 150 mm, Shimadzu) according to the method of Tomiya et al. (1987); (2) on an amide-adsorption, TSK gel Amide-80 (4.6 × 250 mm, TOSOH) according to the method of Tomiya et al. (1988).

Exoglycosidase Digestion of Oligosaccharides. About 10 pmol of each pyridylamino oligosaccharide was separately digested with each glycosidase, at 37 °C for 15 h: β-galactosidase (5 milliunits) in 15 μL of 0.1 M citrate–phosphate buffer (pH 4.1), β-*N*-acetylhexosaminidase (20 milliunits) in 15 μL of 0.1 M citrate–phosphate buffer (pH 5.0), and α-L-fucosidase (20 milliunits) in 15 μL of 0.1 M citrate–phosphate buffer (pH 5.0).

Other Experimental Procedures. For the hydrazinolysis of the erythropoietin, 1 mg of r-EPO (BHK) was hydrazinolyzed with 1 mL of anhydrous hydrazine at 100 °C for 10 h (Takasaki et al., 1982). Liberated oligosaccharides were N-acetylated and pyridylaminated. For the methylation analysis, the pyridylaminated oligosaccharide (about 50 nmol each) separated by HPLC was methylated by the method of Hakomori (1964). The hydrolysate of permethylated product was reduced with $NaBD_4$ and analyzed as described previously (Endo et al., 1979) on a JEOL-DX300 gas chromatograph–mass spectrometer.

^{1}H NMR Measurements. Prior to NMR measurements, pyridylamino derivatives of each oligosaccharide (about 10–100 μg each as neutral sugar) isolated by HPLC were desalted by gel filtration on a Sephadex G-15 column. Samples were dissolved in 99.8% D_2O, lyophilized, and dissolved again in 99.8% D_2O at concentrations of 30–300 μM. NMR measurements were made on a Bruker AM-400 spectrometer operating at 400 MHz in the Fourier transform mode. Measurements were made at 23 and 60 °C. Typically, 2000 transients were accumulated for each measurement. Chemical shifts are expressed in parts per million (ppm) from internal DSS but were actually measured with reference to internal acetone. The chemical shift of acetone in reference to internal DSS was determined to be 2.216 and 2.213 ppm in D_2O at 23 and 60 °C, respectively. Chemical shift values reported are accurate to 0.002 ppm. For the present NMR study, the pyridylamino derivatives of oligosaccharides were used. We

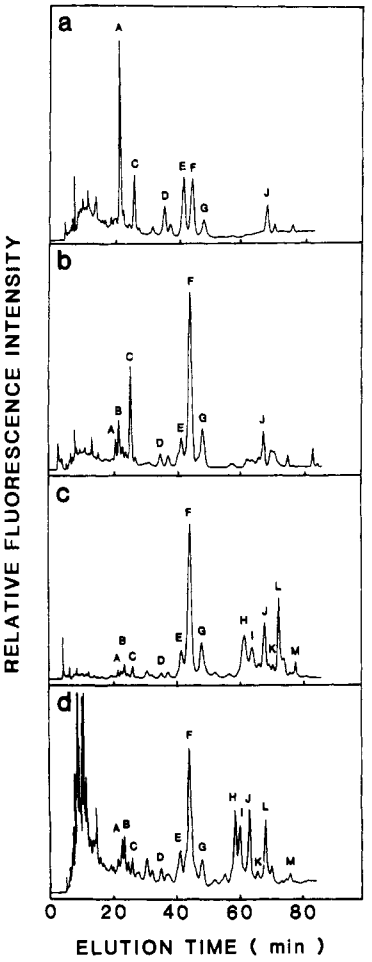


FIGURE 1: Comparison of HPLC profiles obtained on an ODS silica column for pyridylaminated asialooligosaccharides from u-EPO and r-EPO (BHK). (a and b) Oligosaccharide derivatives obtained from two different samples of the u-EPO; (c) oligosaccharide derivatives obtained from r-EPO (BHK); (d) the hydrazinolysis procedure was carried out instead of the glycopeptidase digestion. Extra peaks that emerged before peak A are probably due to the destruction of any oligosaccharide during hydrazinolysis of the protein. The structures of fractions A–M were determined and are as summarized in Figure 3.

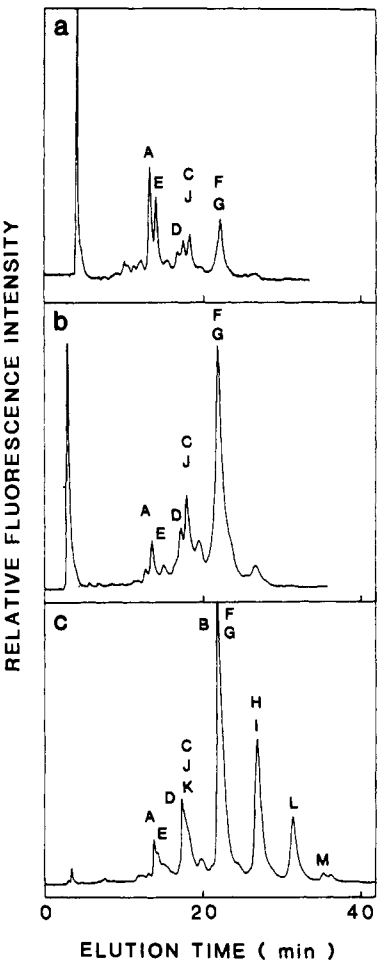


FIGURE 2: Comparison of HPLC profiles obtained on an amide–silica column for pyridylaminated asialooligosaccharides from u-EPO and r-EPO (BHK). Oligosaccharide samples in the panels 2a–c were the same as those in panels a–c of Figure 1, respectively.

have previously shown that signals of residues other than GlcNAc-2 and Man-3 do not show any significant chemical shift changes upon modification of GlcNAc-1 (Takahashi et al., 1986).

Results and Discussion

Sugar Composition of u-EPO and r-EPO (BHK). The sugar composition of each erythropoietin was determined as described previously (Goto et al., 1988). The mole per mole contents of *N*-acetylhexosamine, mannose, fucose, galactose, and sialic acid were as follows: u-EPO, 22.1, 8.1, 2.6, 13.3, and 10.7; r-EPO (BHK), 28.9, 10.1, 3.3, 15.7, and 18.7. It is noted that r-EPO (BHK) possesses a higher content of sialic acid.

Isolation of Oligosaccharides. Asialooligosaccharides were released by sequential digestion of asialo-EPO with pepsin and *N*-oligosaccharide glycopeptidase (almond). It was confirmed on the basis of the results of the sugar content determination that less than about 10% of the total carbohydrates of the original u- and r-EPO (BHK) remain undigested by this procedure. We also prepared another sample of N-linked oligosaccharides from r-EPO (BHK) by hydrazinolysis. The oligosaccharide profile on HPLC obtained by hydrazinolysis of r-EPO (BHK) (Figure 1d) was compared with those obtained by the glycopeptidase digestion (Figure 1c). Spectra c and d of Figure 1 clearly show that the glycopeptidase digestion does not result in any selective release of particular sugars. We therefore used the glycopeptidase digestion method because it is simpler and more suitable for the preparation of large amounts of purified oligosaccharide samples for NMR measurements.

Comparisons of the Distribution of Oligosaccharides among Several Erythropoietins. The 14 samples of u-EPO were collected randomly from a group of aplastic anemic patients. The samples were arbitrary divided into two groups of seven samples. Each of the five r-EPO samples was prepared from a single clone of EPO-producing cells. Oligosaccharide derivatives obtained from two and five different samples of the u-EPO and r-EPO (BHK) proteins, respectively, were subjected to HPLC analyses on an ODS silica column. As Figure 1a,b shows, significant degree of variety exist among u-EPO oligosaccharide patterns. By contrast, there is no significant difference among r-EPO (BHK) oligosaccharide patterns. Figure 1c shows one of the five similar patterns which were obtained from five different samples of r-EPO (BHK) proteins. Figure 2 shows HPLC patterns obtained for u-EPO and r-EPO (BHK) with an amide–silica column. This chromatography fundamentally separates oligosaccharides by their sizes as described previously (Tomiya et al., 1988). Since the saccharides with smaller numbers of *N*-acetyllactosamine units elute earlier than those with higher numbers of *N*-acetyllactosamine units in the amide–silica column, it clearly shows

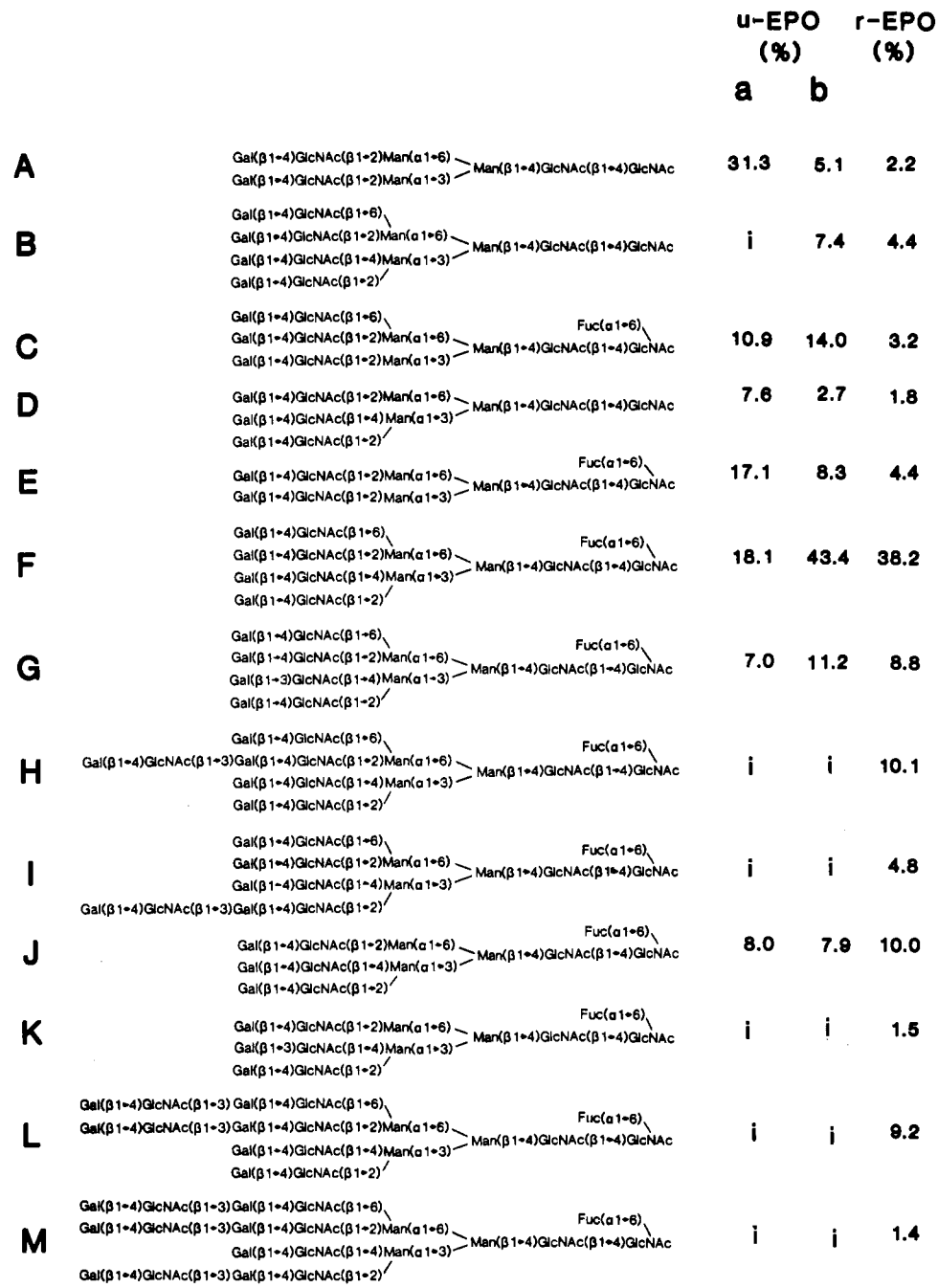


FIGURE 3: Proposed structures of asialo N-linked oligosaccharides obtained from u-EPO and r-EPO (BHK). "i" means insufficient material for detection.

that the molecular sizes of a part of the oligosaccharides of r-EPO (BHK) (Figure 2c) are larger than those of u-EPO. The structure of each of the oligosaccharide derivatives A–M separated completely by HPLC with ODS silica (Figure 1) was characterized on the basis of the results obtained by a combination of sequential exoglycosidase digestion and HPLC analysis. Furthermore, in the case of r-EPO (BHK), ^{1}H NMR measurements and methylation analyses were carried out as described below. Assignments of structures to each of these oligosaccharides are summarized in Figure 3.

Structure of Fraction F, Which Is the Main Component of both u-EPO and r-EPO (BHK). ^{1}H NMR spectral data for the H-1 and acetyl groups clearly indicate that the chemical shifts of fraction F and a standard tetraantennary oligosaccharide from α_1-acid glycoprotein were all in good agreement except a fucose residue which does not exist in the latter (Table I). Furthermore, we compared the elution position of fraction F and the compounds derived from fraction F by sequential glycosidase digestion with those of a standard tetraantennary oligosaccharide with fucose residue obtained from porcine pancreatic lipase, on HPLC with ODS silica and amide–silica columns (Figure 4). The elution positions for fraction F and its digestion products are coincident with those of standard compounds. These results indicate that fraction F is a tetraantennary oligosaccharide with fucose (Figure 3).

Structures of Other Common Oligosaccharides in u-EPO and r-EPO (BHK): Fractions A–E and J. The structures of fractions A–E and J were analyzed by procedures analogous to those described for fraction F and are summarized in Figure 3. Chemical shift data obtained for fractions C, E, and J are summarized in Table I. The chemical shifts for fraction E and "compound H of immunoglobulin G" (Takahashi et al., 1987), which has been shown to be a biantennary oligosaccharide with a fucose residue, were in good agreement.

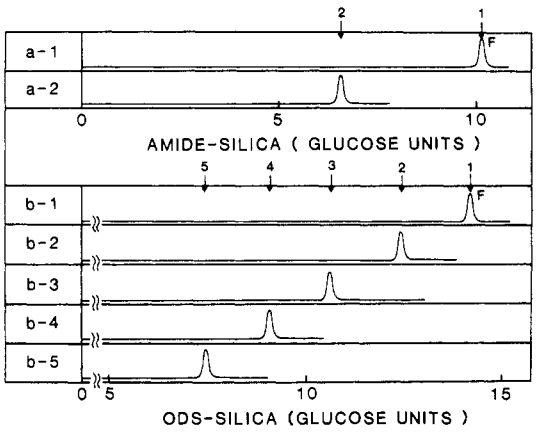
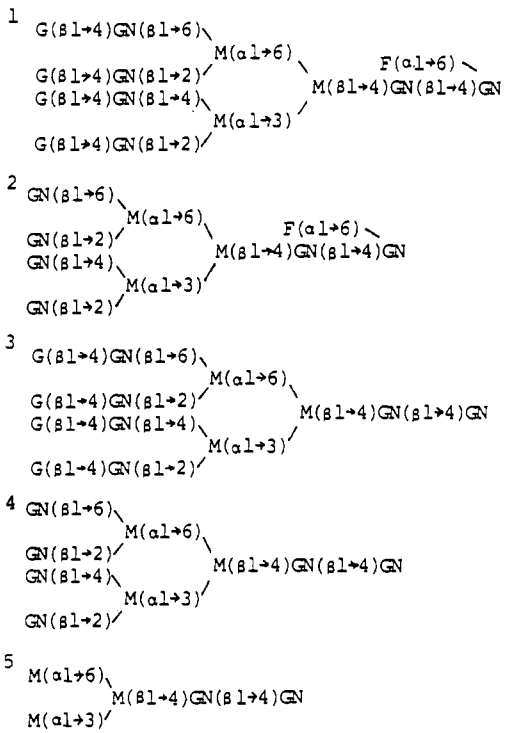


FIGURE 4: HPLC analyses of exoglycosidase digestion of fraction F from u-EPO and r-EPO (BHK). (a and b) Elution positions on amide–silica and ODS silica columns, respectively. (a-1) Intact fraction F; (a-2) fraction F digested with β-galactosidase (C. *lamps*); (b-1) intact fraction F; (b-2) fraction F digested with β-galactosidase; (b-3) fraction F digested with α-L-fucosidase (bovine kidney); (b-4) fraction F sequentially digested with α-L-fucosidase and β-galactosidase; (b-5) fraction F sequentially digested with α-L-fucosidase, β-galactosidase, and β-*N*-acetylglucosaminidase (jack bean). Arrows 1–5 indicate the elution positions of the standard pyridylamino derivatives:



In the standard tetraantennary oligosaccharide from α_1-acid glycoprotein, the H-1 chemical shifts for GlcNAc-7 and GlcNAc-7′ are both 4.541 ppm. However, the chemical shifts for the acetyl groups are different for GlcNAc-7 (2.065 ppm) and GlcNAc-7′ (2.032 ppm). It is shown that fraction C has only GlcNAc-7′ (2.034 ppm) and fraction J has only GlcNAc-7 (2.065 ppm). In the case of α_1-acid glycoprotein, the H-1 chemical shift for GlcNAc-5′ is 4.583 ppm. It is shown that the chemical shift for GlcNAc-5′ of fraction J is shifted to 4.569 ppm, because of the effect of the absence of GlcNAc-7′. These NMR data indicate that both fractions C and J are triantennary oligosaccharides with a fucose residue. It is noted that the difference of the linkage position of an *N*-acetylglucosamine residue attached to the trimannosyl core causes the large difference of the elution position on the HPLC

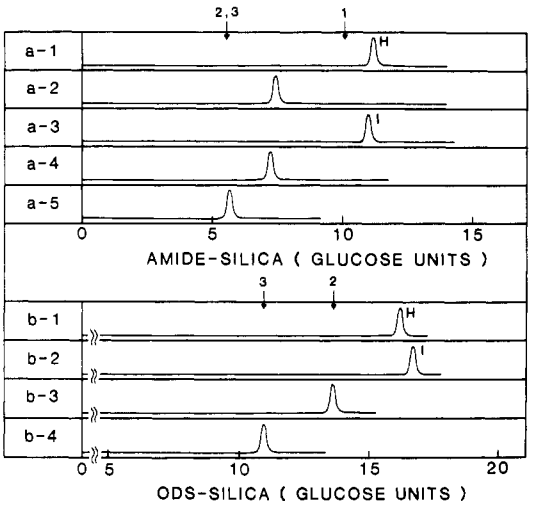
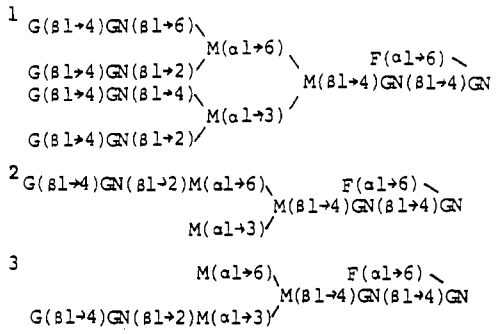


FIGURE 5: HPLC analyses of exoglycosidase digestion of fractions H and I from r-EPO (BHK). (a and b) Elution positions on amide–silica and ODS silica columns, respectively. (a-1) Intact fraction H; (a-2) fraction H digested with β-galactosidase; (a-3) intact fraction I; (a-4) fraction I digested with β-galactosidase; (a-5) fractions H and I sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase; (b-1) intact fraction H; (b-2) intact fraction I; (b-3) fraction H sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase; (b-4) fraction I sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase. Arrows 1–3 indicate the elution positions of the standard pyridylamino derivatives:



profile obtained with an ODS silica column (see Figure 1).

N-Acetyllactosamine-Type Oligosaccharides with Gal-(β1→4)GlcNAc Repeating Units: Fractions H, I, L, and M. The NMR measurements indicate that fractions H and I are a series of compounds of the fucose-containing tetraantennary *N*-acetyllactosamine type that share one repeating unit, Gal-GlcNAc (Table I). However, the chemicl shifts of H-1 protons of all GlcNAc and Gal residues and the chemical shifts for the acetyl group of all GlcNAc residues gave no significant information about the linkage position of each repeating unit.

After digestion of fractions H and I with β-galactosidase, the elution positions of the products from both fractions were the same on an amide–silica column, where the decrease of the molecular size corresponds to four glucose units (Figure 5). After a sequential digestion with β-galactosidase and β-*N*-acetylglucosaminidase, the elution positions of the reaction products of fractions H and I were again the same on an amide–silica column as was that of $Gal_1GlcNAc_1Man_3GlcNAc_1Fuc_1GlcNAc$-PA. However, as shown on the HPLC profiles obtained with an ODS silica column, the elution positions of two products are significantly different. This shows that fraction H has changed to an oligosaccharide with the structure

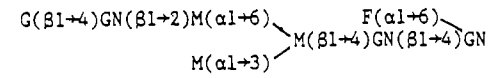
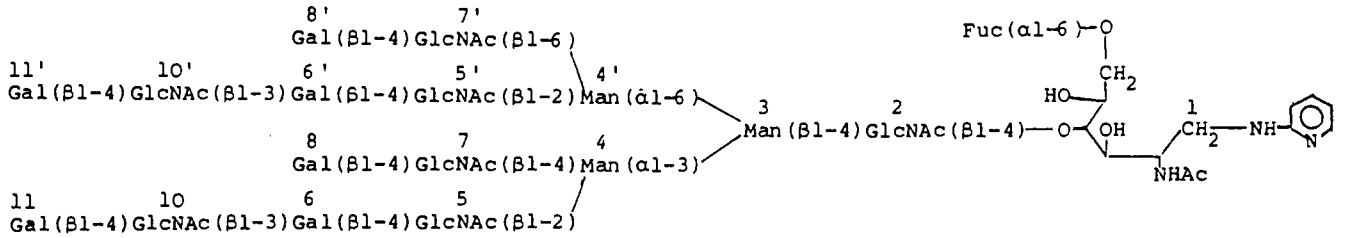


Table I: Chemical Shifts of Anomeric Protons and Methyl Protons for the Pyridylamino Derivatives of Oligosaccharides of Human Urinary and Recombinant Erythropoietins and α_1-Acid Glycoprotein

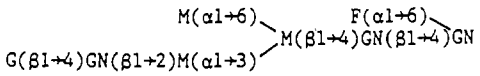


	chemical shifts[a] of anomeric protons														
compd	Man-3	Man-4	Man-4′	Glc-NAc-5	Glc-NAc-5′	Glc-NAc-7	Glc-NAc-7′	Glc-NAc-10	Glc-NAc-10′	Gal-6	Gal-6′	Gal-8	Gal-8′	Gal-11	Gal-11′	Fuc
α_1-acid GP	(4.739)	(5.125)	(4.853)	4.563	4.583	4.541	4.541			4.460	4.460	4.460	4.473			
EPO																
C	(4.745)	(5.121)	(4.852)	4.560	4.577		4.541			4.461	4.461		4.473			(4.852)
E	(4.749)	(5.118)	(4.842)	4.570	4.570					4.461	4.461					(4.907)
F	(4.741)	(5.122)	(4.850)	4.559	4.577	4.538	4.538			4.456	4.456	4.456	4.470			(4.850)
G	(4.738)	(5.127)	(4.855)	4.540	4.562	4.562[c]	4.540			4.457	4.457	4.436[c]	4.473			(4.855)
H	(4.738)	(5.122)	(4.848)	4.560	4.578	4.538	4.538		4.538	4.453	4.446	4.453	4.471		4.471	(4.848)
I	(4.737)	(5.128)	(4.855)	4.559	4.577	4.537	4.537	4.537		4.458	4.458	4.458	4.471	4.471		(4.855)
J	(4.738)	(5.112)	(4.849)	4.556	4.569	4.537				4.459	4.459	4.459				(4.906)
L	(4.735)	(5.123)	(4.848)	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	(4.848)

	chemical shifts[a] of methyl protons						
compd	GlcNAc-5	GlcNAc-5′	GlcNAc-7	GlcNAc-7′	GlcNAc-10	GlcNAc-10′	Fuc
α_1-acid GP	2.047	2.032	2.065	2.032			
EPO							
C	2.051	2.034		2.034			1.170
E	2.044	2.038					1.166
F	2.047	2.033	2.071	2.033			1.168
G	2.050	2.030	2.061[c]	2.030			1.180
H	2.047	2.027	2.068	2.027		2.027	1.171
I	2.047	2.028	2.069	2.028	2.028		1.173
J	2.043	2.036	2.065				1.169
L	2.046	2.026	2.068	2.026	2.026	2.026	1.173

[a] Chemical shifts are expressed in ppm from internal DSS but were actually measured with internal acetone (2.216 ppm in D_2O at 23 °C). Values in parentheses were measured at 60 °C with internal acetone (2.213 ppm in D_2O). [b] The signal was not detectable due to the limited amount of sample. [c] Gal-8(β1→3)GlcNAc-7.

and fraction I to another monoantennary oligosaccharide with the structure

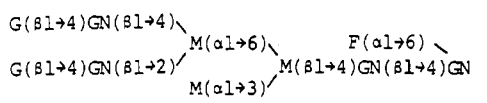


The two monoantennary oligosaccharides described above can be differentiated completely on an ODS silica column (Tomiya et al., 1987), indicating that the repeating unit is attached to Gal-6′ in fraction H and to Gal-6 in fraction I.

The results of the methylation analyses of fractions H and I show that both fractions H and I have four nonreducing terminal galactose residues and one galactose residue substituted at position 3, which is a characteristic substitution in the linkage position attached to each repeating unit (data not shown).

Fractions L and M were analyzed in a similar manner as above. Six kinds of methyl proton resonances due to GlcNAc-5, -5′, -7, -7′, -10, and -10′ are shown for fraction L (Table I). The NMR data clearly indicate that fraction L has two Gal-GlcNAc repeating units, although the linkage positions attached to the galactose residues are not shown.

HPLC experiments on an amide–silica column show that the molecular sizes for fractions L and M are larger by two and three Gal-GlcNAc repeating units, respectively, than that of fraction F (tetraantennary with fucose) (Figure 6). It was confirmed that β-galactosidase digestion removes four galactose residues from both fractions L and M. Furthermore, it was also confirmed that after a sequential digestion with β-galactosidase and β-*N*-acetylglucosaminidase the molecular sizes of the products from fractions L and M become same as those of biantennary (with fucose) and triantennary (with fucose) oligosaccharides, respectively. This result shows that fractions L and M have two and three Gal-GlcNAc repeating units, respectively. In order to determine the linkage position of the repeating units in fraction L, we compared the elution position on an ODS silica column for the final products of the sequential digestion of the above two glycosidases with those of standard biantennary oligosaccharides. Since fraction L resulted in the oligosaccharide with the structure



these results show that the repeating units are attached to Gal-6′ and Gal-8′ in fraction L (Figure 3). In the case of fraction M, the elution position on an ODS silica column for the final product of the sequential digestion by the above two glycosidases is in agreement with that of fraction C (Figure 6). These results show that the linking positions of the repeating units are Gal-6, Gal-6′, and Gal-8′ in fraction M (Figure 3).

In a comparison of the above results obtained with BHK cells to those with CHO cells (Sasaki et al., 1987), there are a few differences in the linking positions of *N*-acetyllactosaminyl repeats. The posttranslational events (glycosylation) could be different in the two cell lines. Several factors such as the presence, or absence, of specific glycosyl trans-

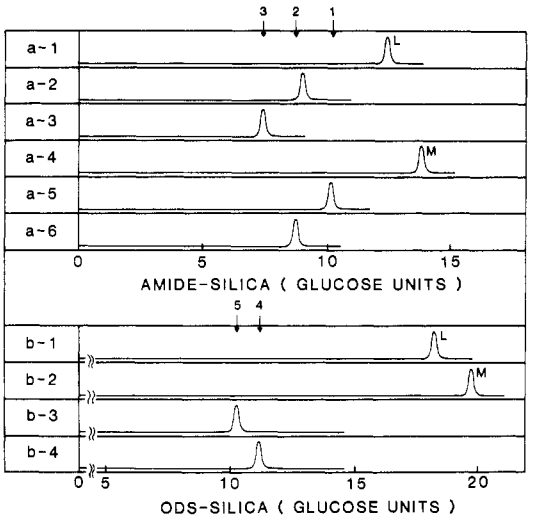
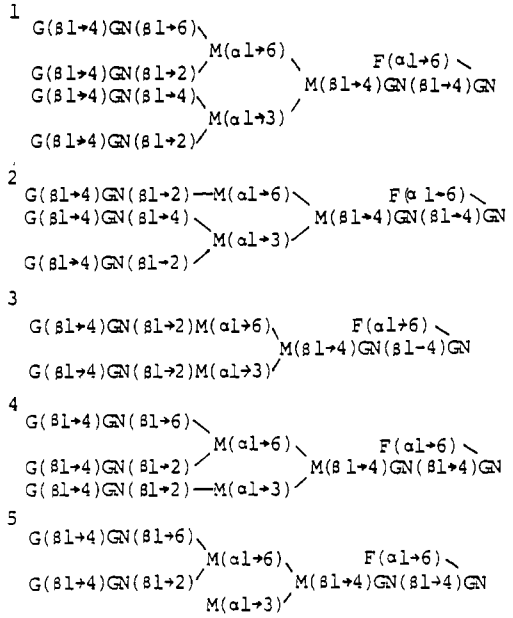


FIGURE 6: HPLC analyses of exoglycosidase digestion of fractions L and M from r-EPO (BHK). (a and b) Elution positions on amide–silica and ODS silica columns, respectively. (a-1) Intact fraction L; (A-2) fraction L digested with β-galactosidase; (a-3) fraction L sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase; (a-4) intact fraction M; (a-5) fraction M digested with β-galactosidase; (a-6) fraction M sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase. (b-1) intact fraction L; (b-2) intact fraction M; (b-3) fraction L sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase; (b-4) fraction M sequentially digested with β-galactosidase and β-*N*-acetylglucosaminidase. Arrows 1–5 indicate the elution positions of the standard pyridylamino derivatives:



ferases could affect the structures of the oligosaccharides formed.

Fractions G and K: N-Acetyllactosamine-Type Oligosaccharides with a Gal(β1→3)GlcNAc Sequence. A ^{1}H NMR spectrum of fraction G is reproduced in Figure 7. The ^{1}H NMR spectral data for the H-1 group of fraction G clearly indicate that, although fraction G represents a tetraantennary oligosaccharide with a fucose residue, an unusual linkage exists for GlcNAc-7. The chemical shift for the acetyl group of GlcNAc-7 is shifted significantly from 2.071 ppm (for fraction F which is a typical tetraantennary oligosaccharide) to 2.061 ppm. The H-1 chemical shift assigned for GlcNAc-7 of fraction G is shifted from 4.538 (fraction F) to 4.562 ppm. The chemical shift for Gal-8 is shifted from 4.456 (fraction F) to 4.436 ppm. These results are reminiscent of the results

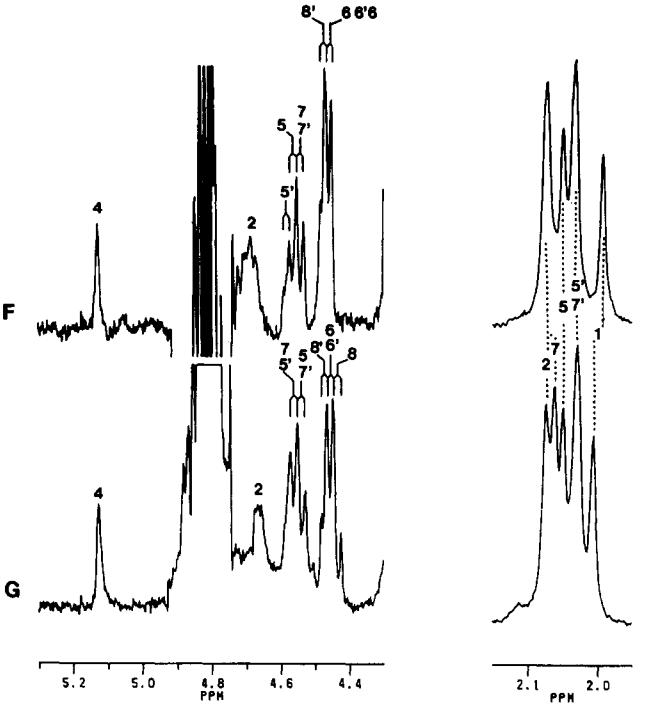


FIGURE 7: The 400-MHz ^{1}H NMR spectra of anomeric (left) and methyl (right) protons of fractions F and G from r-EPO (BHK). Assignments: 4, Man-4; 2, GlcNAc-2; 5, GlcNAc-5; 5′, GlcNAc-5′; 6, Gal-6′; 6′, Gal-6′; 7, GlcNAc-7; 7′, GlcNAc-7′; 8, Gal-8; 8′, Gal-8′. The free induction decay was recorded with 16K data points and a spectral width of ±2500 Hz. Typically, 1000 transients were acquired, and a line broadening of 0.3 Hz was applied prior to Fourier transformation. The probe temperature was 23 °C.

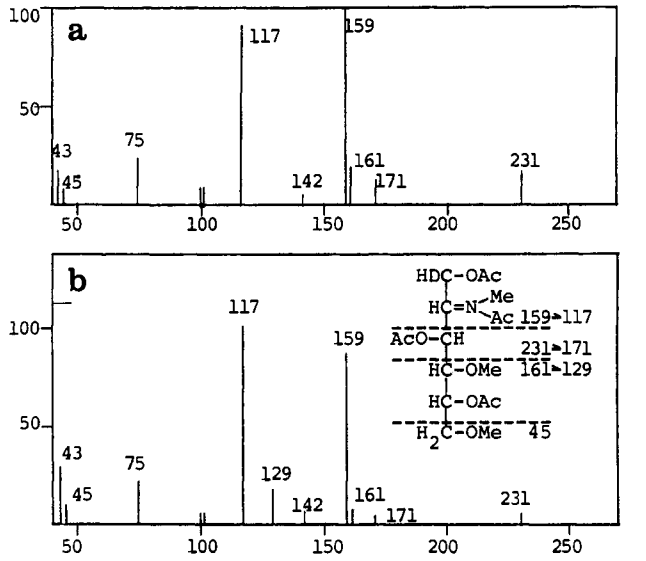


FIGURE 8: Mass spectra of partially O-methylated 2-(*N*-methylacetamido)-2-deoxyglucitol acetates: (a) from fraction G from r-EPO; (b) acetate of 4,6-di-*O*-methyl from Galβ(1,3)GlcNAc-TRI from fetuin.

obtained from the oligosaccharides of fetuin, "Galβ(1,4)-GlcNAc-TRI" and "Galβ(1,3)GlcNAc-TRI" (Townsend et al., 1986). In the case of fetuin, a downfield shift of 0.027 ppm and an upfield shift of 0.026 ppm were reported in the H-1 of GlcNAc-7 and Gal-8, respectively. We therefore conclude that the shifts observed for fraction G were due to the β1→3 substitution of GlcNAc-7 with Gal-8.

Fraction G and oligosaccharide Galβ(1,3)GlcNAc-TRI from fetuin were subjected to methylation analysis. As summarized in Figure 8, both fractions G and Galβ(1,3)-GlcNAc-TRI gave essentially the same spectra for the acetate

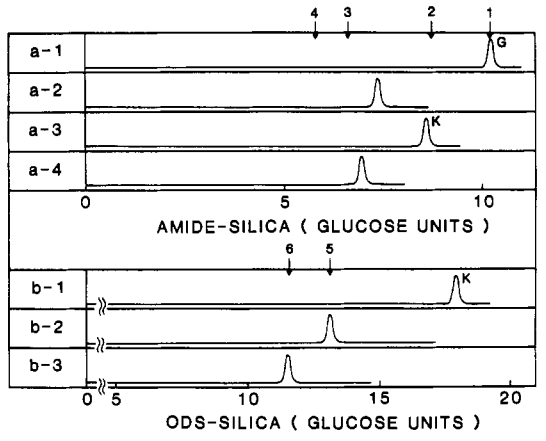
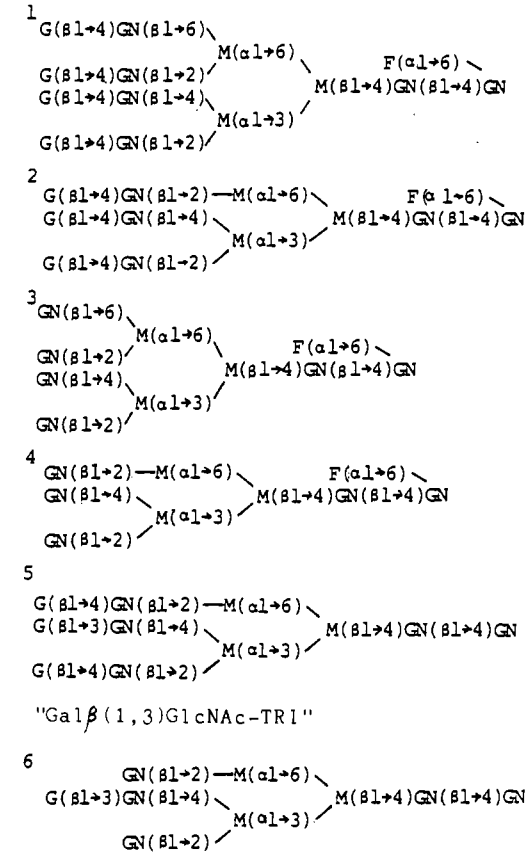


FIGURE 9: HPLC analyses of exoglycosidase digestion of fractions G and K from u-EPO and r-EPO (BHK). (a and b) Elution positions on amide–silica and ODS silica columns, respectively; (a-1) Intact fraction G; (a-2) fraction G digested with β-galactosidase; (a-3) intact fraction K; (a-4) fraction K digested with β-galactosidase; (b-1) intact fraction K; (b-2) fraction K digested with α-L-fucosidase; (b-3) fraction K sequentially digested with α-L-fucosidase and β-galactosidase (*C. lampas*). Arrows 1–6 indicate the elution positions of the standard pyridylamino derivatives:



of 4,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol. The results clearly show that fraction G possesses four GlcNAc residues substituted at position 4 and one GlcNAc residue substituted at position 3. The molecular sizes of fractions G and K as observed on an amide–silica column (Figure 9) are the same as those of fraction F (tetraantennary with fucose) and fraction J (triantennary with fucose), respectively. This result indicates that the molecular sizes of fractions G and K are the same as those of $Gal_4GlcNAc_4Man_3GlcNAc(Fuc)$-GlcNAc-PA and $Gal_3GlcNAc_3Man_3GlcNAc(Fuc)GlcNAc$-PA, respectively. After digestion with β-galactosidase from *Charonia lampas*, which does not cleave the Galβ1→3 linkage, three of four and two of three galactose residues were removed from fractions G and K, respectively (Figure 9). Because of the limited amount of fraction K, the structure was analyzed with a combination of HPLC and sequential exoglycosidase digestion. Defucosylated fraction K and standard Galβ-(1,3)GlcNAc-TRI (Townsend et al., 1986) revealed the same behavior on HPLC.

In so far as we know, a tetraantennary oligosaccharide (with and without fucose) containing the Gal(β1→3)GlcNAc sequence in the outer chain moiety has not been isolated. In the present experiment we have shown that this unusual oligosaccharide (fraction G) is one of the most abundant components in both u-EPO and r-EPO (BHK). It should also be emphasized that the triantennary sugar chain (with fucose) containing Gal(β1→3)GlcNAc (fraction K) exists in r-EPO (BHK). The biological significance of the presence in EPO of the Galβ1→3 linkage containing tetraantennary oligosaccharide is not clear at present. EPO is a hormone whose level in blood has to be kept constant. Townsend et al. (1987) reported that Galβ(1,3)GlcNAc-TRI has much lower affinity than Galβ(1,4)GlcNAc-TRI to rabbit hepatocytes; i.e., the K_d for Galβ(1,4)GlcNAc-TRI and rabbit hepatocytes is 4.4 nM, and the K_d for Galβ(1,3)GlcNAc-TRI and rabbit heatocytes is 305 nM. We therefore suggest that the Gal(β1→3)GlcNAc sequence in oligosaccharides of u-EPO plays a physiological role to prevent the excess trap of EPO to hepatocytes.

ACKNOWLEDGMENTS

N.T. is greatly indebted to Dr. Ryo Tanaka of Nagoya City University for his helpful discussion. We thank Y. Wada for her excellent technical assistance. We gratefully acknowledge generous considerations by Dr. H. Hanssum and S. Ueki of Bruker Japan for the use of their WM-400 spectrometer.

REFERENCES

Dordal, M. S., Wang, F. F., & Goldwasser, E. (1985) *Endocrinology (Baltimore) 116*, 2293–2299.

Egrie, J. C., Strickland, T. W., Lane, J., Aoki, K., Cohen, A. M., Smalling, R., Trail, G., Lin, F. K., Browne, J. K., & Hines, D. K. (1986) *Immunobiology (Stuttgart) 172*, 213–224.

Endo, Y., Yamashita, K., Tachibana, Y., Tojo, S., & Kobata, A. (1979) *J. Biochem. (Tokyo) 85*, 669–679.

Goto, M., Akai, K., Murakami, A., Hashimoto, C., Tsuda, E., Ueda, M., Kawanishi, G., Takahashi, N., Ishimoto, A., Chiba, H., & Sasaki, R. (1988) *Bio/Technology 6*, 67–71.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Hase, S., Ibuki, T., & Ikenaka, T. (1984) *J. Biochem. (Tokyo) 95*, 197–203.

Hase, S., Koyama, S., Daiyasu, H., Takemoto, H., Hara, S., Kobayashi, Y., Kyogoku, Y., & Ikenaka, T. (1986) *J. Biochem. (Tokyo) 100*, 1–10.

Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S. D., Kaufman, R. J., Mufson, A., Seehra, J., Jones, S. S., Hewick, R., Fritsh, E. F., Kawakita, M., Shimizu, T., & Miyake, T. (1985) *Nature (London) 313*, 806–810.

Miyake, T., Kung, C. K.-H., & Goldwasser, E. (1977) *J. Biol. Chem. 252*, 5558–5564.

Nishibe, H., & Takahashi, N. (1981) *Biochim. Biophys. Acta 661*, 274–279.

Nishigaki, M., Yamashita, K., Matsuda, I., Arashima, S., & Kobata, A. (1978), *J. Biochem. (Tokyo) 84*, 823–834.

Powell, J. S., Berkner, K. L., Lebo, R. V., & Adamson, J. W. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6465–6469.

Sasaki, H., Bothner, B., Dell, A., & Fukuda, M. (1987) *J. Biol. Chem. 262*, 12059–12076.

Takahashi, N., Hotta, T., Ishihara, H., Mori, M., Tejima, S., Bligny, R., Akazawa, T., Endo, S., & Arata, Y. (1986) *Biochemistry 25*, 388–395.
Takahashi, N., Ishii, I., Ishihara, H., Mori, M., Tejima, S., Jefferis, R., Endo, S., & Arata, Y. (1987) *Biochemistry 26*, 1137–1144.
Takasaki, S., Mizuochi, T., & Kobata, A. (1982) *Methods Enzymol. 83*, 263–268.
Tomiya, N., Kurono, M., Ishihara, H., Tejima, S., Endo, S., Arata, Y., & Takahashi, N. (1987) *Anal. Biochem. 163*, 489–499.
Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., & Takahashi, N. (1988) *Anal. Biochem. 171*, 73–90.
Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry 25*, 5716–5725.
Yanagawa, S., Hirade, K., Ohnota, H., Sasaki, R., Chiba, H., Ueda, M., & Goto, M. (1984) *J. Biol. Chem. 259*, 2707–2710.
Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T., & Kobata, A. (1981) *J. Biol. Chem. 256*, 8476–8484.

Effects of Fusion Temperature on the Lateral Mobility of Sendai Virus Glycoproteins in Erythrocyte Membranes and on Cell Fusion Indicate That Glycoprotein Mobilization Is Required for Cell Fusion†

Benjamin Aroeti and Yoav I. Henis*
Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
Received December 3, 1987; Revised Manuscript Received March 17, 1988

ABSTRACT: In order to investigate the requirement for lateral mobilization of viral envelope glycoproteins on the cell surface in the induction of cell–cell fusion, we employed fluorescence photobleaching recovery to study the effect of the fusion temperature on the lateral mobilization of Sendai virus glycoproteins in the human erythrocyte membrane. As the fusion temperature was reduced below 37 °C (to 31 or 25 °C), the rates of virus–cell fusion, the accompanying hemolysis, and cell–cell fusion were all slowed down. However, the plateau (final level) after the completion of fusion was significantly reduced at lower fusion temperatures only in the case of cell–cell fusion, despite the rather similar final levels of virus–cell fusion. A concomitant decrease as a function of the fusion temperature was observed in the fraction of cell-associated viral glycoproteins that became laterally mobile in the erythrocyte membrane during fusion, and a strict correlation was found between the level of laterally mobile viral glycoproteins in the cell membrane and the final extent of cell–cell fusion. The accompanying reduction in the lateral diffusion coefficients (D) of the viral glycoproteins (1.4-fold at 31 °C and 1.9-fold at 25 °C, as compared to 37 °C) does not appear to determine the final level of cell–cell fusion, since fusing the cells with a higher amount of virions at 25 °C increased the final level of cell–cell fusion while D remained constant. The results demonstrate that lateral mobilization of the viral glycoproteins in the target cell membrane is not an immediate consequence of viral envelope–cell fusion and support the view that it plays an essential role in the induction of cell–cell fusion by native Sendai virions.

The membrane fusion activities of animal enveloped viruses have been shown to be mediated by specific viral envelope glycoproteins (Poste & Pasternak, 1978; Volsky & Loyter, 1978a; Hsu et al., 1979; White et al., 1983; Florkiewicz & Rose, 1984). Among the paramyxoviruses, which fuse with cell membranes at neutral pH values (Poste & Pasternak, 1978; White et al., 1983), the fusogenic activities of Sendai virus have been the most extensively investigated. The envelope of Sendai virions contains two glycoproteins: the fusion protein F,[1] which is required for virus–cell and cell–cell fusion, and the hemagglutinin-neuraminidase protein (HN), which serves to bind the virions to sialic acid containing membrane components (Poste & Pasternak, 1978; Choppin & Scheid, 1980).

The mechanism of virus–cell and cell–cell fusion is still obscure, although an involvement of hydrophobic interactions between viral glycoproteins and target membranes was proposed (Gething et al., 1978; Hsu et al., 1981; White et al., 1983). Employing fluorescence photobleaching recovery (FPR), we have recently demonstrated that the envelope glycoproteins of native Sendai virions become laterally mobile on the surface of human erythrocytes following fusion and that this mobilization is blocked under conditions that eliminate virus–cell and cell–cell fusion. These findings supported the notion that lateral motion of the viral glycoproteins in the cell membrane may be required for the induction of cell–cell fusion (Henis et al., 1985; Volsky & Loyter, 1978b; Kuroda et al., 1980). However, since both virus–cell and cell–cell fusion were blocked, it was not clear whether the lateral mobilization of

† This work was supported in part by the Fund for Basic Research (administered by the Israel Academy of Sciences and Humanities).
* To whom correspondence should be addressed.

[1] Abbreviations: D, lateral diffusion coefficient; DTT, dithiothreitol; F, fusion protein; FPR, fluorescence photobleaching recovery; HAU, hemagglutinating unit(s); HN, hemagglutinin-neuraminidase protein; PMSF, phenylmethanesulfonyl fluoride; R_f, mobile fraction; RSVE, reconstitute Sendai virus envelopes; R_{18}, octadecylrhodamine B chloride; TMR, tetramethylrhodamine; Tricine, *N*-[tris(hydroxymethyl)methyl]-glycine.

0006-2960/88/0427-5654$01.50/0