Sialylated carbohydrate chains of recombinant human glycoproteins expressed in Chinese hamster ovary cells contain traces of *N*-glycolylneuraminic acid

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HPLC analysis of sialic acids released from recombinant variants of human tissue plasminogen activator, human chimeric plasminogen activator, human erythropoietin, and human follitropin, expressed in Chinese hamster ovary cells, demonstrates for each glycoprotein the presence of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid in a ratio of 97:3. Structural analysis by 500 MHz ^{1}H-NMR spectroscopy, of the enzymatically released N-linked carbohydrate chains of chimeric plasminogen activator and of erythropoietin, showed that α2-3 linked *N*-glycolylneuraminic acid can occur in different *N*-acetyllactosamine type antennary structures.

Recombinant human glycoprotein; Chinese hamster ovary cell; *N*-Glycolylneuraminic acid

1. INTRODUCTION

The accumulating evidence concerning the biological relevance of the carbohydrate part of glycoproteins has highly stimulated the interest in biochemical and pharmaceutical aspects of these biopolymers [1-3]. In particular, it has activated the discussion with respect to genetically engineered proteins expressed in heterologous cell types with or without glycosylation machineries [4,5]. Because the cellular glycosylation machinery can vary among different cell lines, different glycosylation patterns of glycoproteins can be expected. Based on the present knowledge about primary carbohydrate structures of recombinant human glycoproteins, the Chinese hamster ovary (CHO) cells seem to be preferred [6].

In the framework of our program on the microanalysis of (recombinant) glycoprotein glycans, we have discovered that CHO cells can generate asparagine-linked carbohydrate chains containing *N*-glycolylneuraminic acid (Neu5Gc), as illustrated here for 4 recombinant human glycoproteins.

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Fuc, fucose; CHO, Chinese hamster ovary; tPA, tissue plasminogen activator; k$_2$tuPA, recombinant chimeric plasminogen activator; EPO, erythropoietin; FSH, follicle stimulating hormone, follitropin; hCG, human chorionic gonadotropin; PNGase-F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; DMB, 1,2-diamino-4,5-methylene-dioxybenzene.

2. MATERIALS AND METHODS

2.1. *Materials*

Commercially available recombinant human tissue plasminogen activator (tPA), expressed in CHO cells (Actilyse), was obtained from Thomae (Biberach an der Riss, FRG). CHO-cell derived recombinant human chimeric plasminogen activator (k$_2$tuPA) was a gift of Ciba-Geigy Ltd (Basel, Switserland), CHO-cell derived recombinant human erythropoietin (EPO) was a gift of Organon Teknika BV (Boxtel, The Netherlands), and CHO-cell derived recombinant human follitropin (FSH) was a gift of Diosynth BV (Oss, The Netherlands). Human serotransferrin and bovine fibrinogen were obtained from Sigma. Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) from *Flavobacterium meningosepticum* was purchased from Boehringer Mannheim.

2.2. *Liberation of N-linked carbohydrate chains*

The N-linked carbohydrate chains of k$_2$tuPA and EPO were released by treatment with non-immobilized PNGase-F essentially as described [7,8]. In case of EPO a complete liberation of oligosaccharides was obtained without the addition of Non-idet P-40, as indicated by SDS-PAGE. Released carbohydrate chains were collected by gel-permeation chromatography on Bio-Gel P-100 (BioRad) using 50 mM (k$_2$tuPA) or 25 mM (EPO) NH_4HCO_3, pH 7.0, as eluent. In each case, the pooled carbohydrate fraction was separated according to charge by FPLC on Mono Q (Pharmacia), followed by HPLC of the subfractions on Lichrosorb-NH_2 (Chrompack).

2.3. *Analysis of sialic acids*

The analysis of sialic acids was carried out essentially as described [9]. Briefly, aliquots of sialoglycoproteins (0.25-1 mg) in 0.2 ml of 2 M acetic acid were heated for 3 h at 80°C, and released sialic acids were converted with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) into fluorescent derivatives. *N*-Acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) were used as standards. HPLC analysis was carried out on a Kratos Spectroflow 400 HPLC system equipped with a Chromspher C_{18} (Chrompack) reversed-phase column (25 × 0.46 cm) and Kratos 980 fluorescence detector operating at an excitation wavelength of 373 nm, detection emission at wavelengths > 448 nm using a cut-off filter. Elutions were performed

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using acetonitrile: methanol: water (9:7:84, v:v:v) as eluent at a flow rate of 1 ml/min.

2.4. *500 MHz ^{1}H-NMR spectroscopy*

Prior to ^{1}H-NMR spectroscopic analysis, samples were repeatedly treated with ^2H_2O, finally using 99.96 atom % ^2H_2O (MSD isotopes) at p^{2}H 7 and room temperature. 500 MHz ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR spectroscopy, Utrecht University) at a probe temperature of 27 °C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm) [10].

3. RESULTS

3.1. *Analysis of sialic acids*

Conventional sugar analysis using the methanolysis procedure [11] can not discriminate between *N,O*-acylneuraminic acids, because all sialic acids are converted into Neu5Ac. Therefore, an approach has to be incorporated, which is specifically focused on *N,O*-acylneuraminic acids. As has been recently described [9], released sialic acids can be analyzed by reversed-phase HPLC in the fmol region after conversion with 1,2-diamino-4,5-methylenedioxybenzene, yielding highly fluorescent DMB derivatives. In Fig. 1A,B typical examples are depicted for sialic acids released from human serotransferrin and bovine fibrinogen, respectively. The sialic acid patterns are in agreement with literature data, showing only Neu5Ac for human serotransferrin [12] and a mixture of Neu5Gc and Neu5Ac (ratio 63:37) for bovine fibrinogen [8]. In Fig. 1C the HPLC profile of the DMB sialic acids derived from recombinant human FSH is presented, showing peaks at elution positions corresponding with Neu5Gc and Neu5Ac in a ratio of 3:97. Similar patterns were found for recombinant k$_2$tuPA, EPO, and tPA. These findings strongly suggest that the occurrence of Neu5Gc as a constituent of carbohydrate chains in recombinant glycoproteins expressed in normal CHO cells, is a general feature.

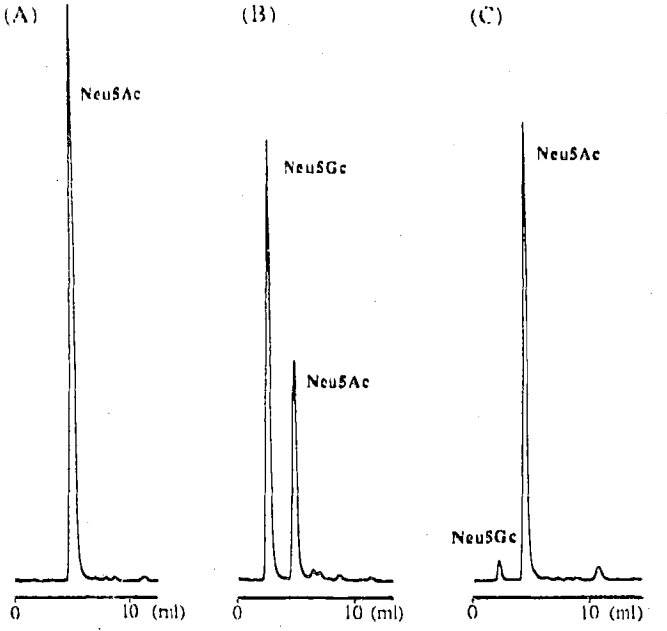


Fig. 1. HPLC Profile of DMB derivatives of sialic acids, released from different glycoproteins, on a Chromspher C_{18} column (25 × 0.46 cm). The elution was carried out with acetonitrile : methanol : water (9 : 7 : 84, v/v/v) at a flow rate of 1 ml/min. (A) human serotransferrin; (B) bovine fibrinogen; (C) recombinant human FSH, expressed in CHO cells.

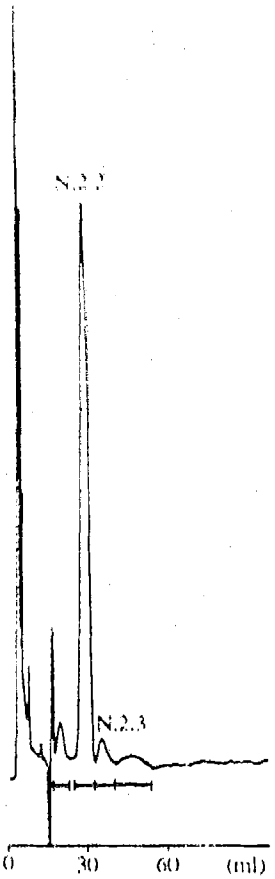


Fig. 2. HPLC Fractionation pattern at 205 nm of the k$_2$tuPA FPLC fraction N2 on a Lichrosorb-NH_2 10 μm column (25 × 0.46 cm). The elution was carried out isocratically with 30 mM K_2HPO_4/KH_2PO_4, pH 7.0: acetonitrile (35 : 65, v/v) at a flow rate of 2 ml/min at ambient temperature. Fractions were collected as indicated.

3.2. *Identification of Neu5Gc-containing N-linked chains in k$_2$tuPA and EPO*

The pool of N-linked carbohydrate chains of k$_2$tuPA, released by PNGase-F, was separated on Mono Q, yielding 4 carbohydrate-positive peaks (N1-N4) having the same retention volumes as mono-, di-, tri-, and tetra-sialo N-type antennary structures, respectively [8]. Each FPLC fraction was further separated by HPLC on Lichrosorb-NH_2. In the context of this paper, the HPLC subfractions N2.2 and N2.3 of FPLC fraction N2 (Fig. 2) will be discussed in more detail. The major fraction N2.2 represents the following conventional disialo diantennary compound:

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 \
Fucα1-6 \
Manβ1-4GlcNAcβ1-4GlcNAc [N2.2]
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 /

its ^{1}H-NMR data fit those of the disialo(Neu5Acα2-3) diantennary oligosaccharide from human chorionic gonadotropin (hCG) [7]. The presence of α2-3 linked Neu5Ac in both antennae is characterized by the typical set of structural-reporter-group signals for NAc at δ = 2.031 ppm, H-3a at δ = 1.797 (Manα1-3 branch)/1.802 (Manα1-6 branch) ppm, and H-3e at δ = 2.758 ppm. The two components present in the minor fraction N2.3 ($R_{N2.3}$ = 1.27 × $R_{N2.2}$) in about equal amounts, turned out to be structural analogues of the Neu5Ac-containing diantennary compound N2.2, namely:

Neu5Gcα2-3Galβ1-4GlcNAcβ1-2Manα1-6 \
Fucα1-6 \
Manβ1-4GlcNAcβ1-4GlcNAc [N2.3A]
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 /

Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 \
Fucα1-6 \
Manβ1-4GlcNAcβ1-4GlcNAc [N2.3B]
Neu5Gcα2-3Galβ1-4GlcNAcβ1-2Manα1-3 /

The ^{1}H-NMR spectrum shows the characteristic structural reporters for the constituting Gal, GlcNAc, Man and Fuc residues, being identical to those of N2.2 (see also [7]). However, as compared to N2.2, the intensity of the NAc signal of Neu5Ac at δ = 2.031 ppm is halved, whereas a new singlet at δ = 4.121 ppm is observed, diagnostic for the *N*-glycolyl group of Neu5Gc [8]. In addition, two sialic acid H-3e double doublets are observed at δ = 2.758 ppm (Neu5Ac) and δ = 2.775 ppm (Neu5Gc), whereas in the H-3a chemical-shift region 4 triplets occur at δ = 1.797 ppm (Neu5Ac, Manα1-3 branch), δ = 1.801 ppm (Neu5Ac, Manα 1-6 branch), δ = 1.813 ppm (Neu5Gc, Manα1-3 branch), and δ = 1.820 ppm (Neu5Gc, Manα1-6 branch). The assignments of the H-3 signals are based on comparison with the related signals in N2.2, and on shift increments going from Neu5Ac to Neu5Gc in α2-6 linked sialic-acid-containing diantennary structures ($\Delta\delta$ = +0.016/0.017 ppm) [8] and in O-linked Siaα2-3Galβ1-3GalNAcol (H-3e, $\Delta\delta$ = +0.013 ppm; H-3a, $\Delta\delta$ = +0.017 ppm) [13]. The relative retention volume of N2.3 with respect to N2.2 is in accordance with the replacement of only one Neu5Ac residue by Neu5Gc (compare with the disialo(α2-6) diantennary carbohydrate chains from bovine fibrinogen [8]).

The pool of N-linked carbohydrate chains of EPO released by PNGase-F was fractionated on Mono Q, yielding 4 carbohydrate-positive peaks (A1–A4) at the same retention positions as mentioned for $k_2$tuPA (see above). Each FPLC fraction was further subfractionated by HPLC on Lichrosorb-NH_2. In the context of this report, the HPLC subfractions A4.4 and A4.5 of FPLC fraction A4 (Fig. 3) will be discussed in more detail. ^{1}H-NMR analysis of subfraction A4.4 demonstrated the presence of the following tetrasialo tetraantennary compound:

Neu5Acα2-3Galβ1-4GlcNAcβ1-6 \
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 \
Fucα1-6 \
Manβ1-4GlcNAcβ1-4GlcNAc [A4.4]
Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 /
Neu5Acα2-3Galβ1-4GlcNAcβ1-4 /

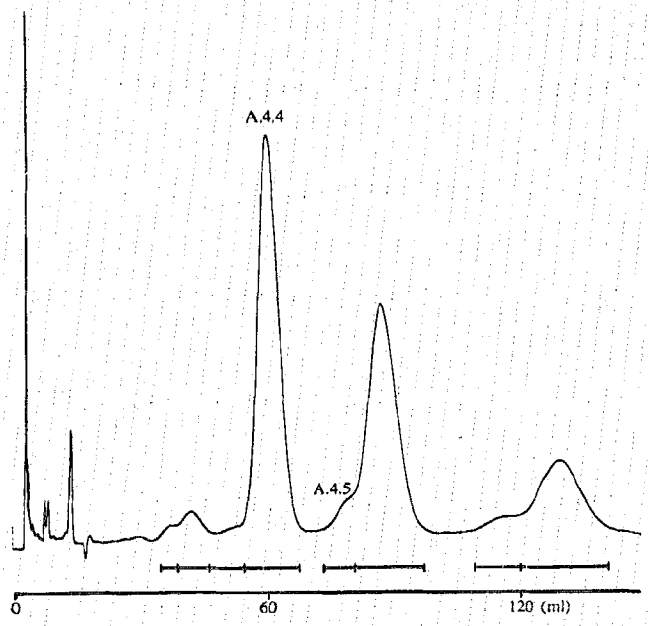
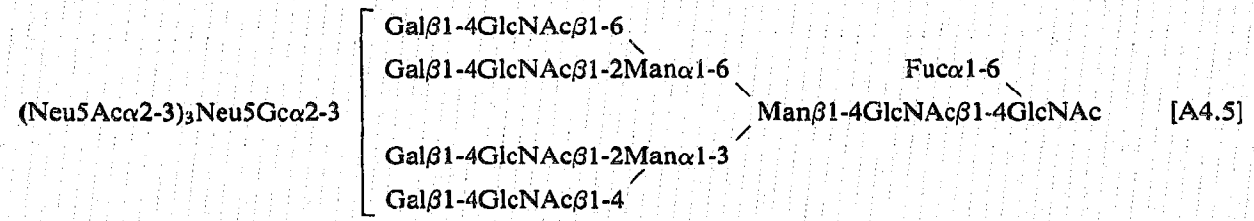


Fig. 3. HPLC Fractionation pattern at 205 nm of the EPO FPLC fraction A4 on a Lichrosorb-NH_2 10 μm column (25 × 0.46 cm). The elution was carried out isocratically with 30 mM K_2HPO_4/KH_2PO_4, pH 7.0 : acetonitrile (37.5 : 62.5, v/v) at a flow rate of 2 ml/min at ambient temperature. Fractions were collected as indicated.

The NMR data of A4.4 are in agreement with those reported for the tetrasialo(Neu5Acα2-3) tetraantennary oligosaccharide from recombinant human FSH [14]. The four α2-3 linked Neu5Ac residues give rise to the characteristic set of structural reporters at δ = 2.031 ppm (4× NAc), δ = 1.804 ppm (4× H-3a), and δ = 2.756 ppm (4× H-3e). Comparison of the ^{1}H-NMR spectra of the subfractions A4.4 and A4.5 shows that the major component in subfraction A4.5 has the same characteristic structural reporters for the constituting Gal, GlcNAc, Man and Fuc residues as those found for A4.4 (see also [14]). However, in case of A4.5 the δ-region covering the H-2 signal for α1-6 linked Man, the H-3 signal of Gal (4×), and the H-5 signal of Fuc, contains an additional singlet at δ = 4.121 ppm, typical for the NGc signal of Neu5Gc [8]. As compared to the relative intensity of the NAc singlet of Neu5Ac in the NAc region of the NMR spectrum of A4.4, the intensity of the NAc singlet in the spectrum of A4.5 is reduced to about 75%. Furthermore, additional signals are present in the sialic acid H-3 regions. Double doublets for H-3e are observed at δ = 2.757 ppm (major, Neu5Ac) and δ = 2.774 ppm (minor, Neu5Gc), whereas triplets are detected for H-3a at δ = 1.804 ppm (major, Neu5Ac) and δ = 1.821 ppm (minor, Neu5Gc). Based on the NMR data and the relative retention volume of A4.5 as compared to A4.4 ($R_{A4.5}$ = 1.3 × $R_{A4.4}$) [8], it can be concluded that overall one of the Neu5Ac residues has been replaced by Neu5Gc in the tetrasialo tetraantennary structure. Whether or not a specific antenna is involved needs to be clarified.



4. DISCUSSION

In view of the literature data on the recombinant variants of human interferon-γ [15], hCG [6], human FSH [14], human interferon-β1 [16–18], bovine lutropin [19], human EPO [20–22], human tPA [23–25], human transforming growth factor-β1 precursor [26], human immunodeficiency virus envelope glycoprotein gp120 [27,28], human CD4 [29,30], and human interleukin-5 [31], it can be concluded that CHO cells can synthesize glycoproteins with (phosphorylated) oligomannose, hybrid, as well as *N*-acetyllactosamine type of structures, depending on the protein involved. The last type comprises mono-, di-, tri-, tri'-, and tetra-antennary glycans. Also poly(*N*-acetyllactosamine) sequences can occur. The terminal Neu5Ac residue is always exclusively linked in α2-3 position to Galβ1-4GlcNAc, indicating the absence of β-Gal α2-6-sialyltransferase activity. It has been shown that the terminal sialylation of N-linked glycans in CHO cells can be altered by expression of this transferase [32]. Structures with bisecting GlcNAc [14], the antennary element GalNAcβ1-4GlcNAc [19], peripheral Fucα1-3GlcNAc [33] and the antennary element Galα-1-3Galβ1-4 [4] are not produced. Recently, also the transfection of a human α1-3 fucosyltransferase gene

into CHO cells has been reported [33]. Sialylated O-linked oligosaccharides as Neu5Acα2-3Galβ1-3GalNAc and Neu5Acα2-3Galβ1-3[Neu5Acα2-6)GalNAc are synthesized in a normal way, including α2-3 as well as α2-6 linked Neu5Ac, as found for the recombinant variants of hCG [6], human EPO [20], human interleukin-2 [34], and human granulocyte-colony-stimulating factor [35]. This means that the CHO cell has normal α-GalNAc α2-6-sialyltransferase activity. Comparison of the carbohydrate chains in those cases in which the native and recombinant glycoproteins have both been analyzed, has made it clear that from a carbohydrate point of view, the CHO cell line is highly attractive.

In this report we have furnished data that recombinant glycoproteins expressed in CHO cells contain carbohydrate chains with a small percentage (~3%) of α2-3 linked Neu5Gc. The occurrence of Neu5Gc in normal human tissue and soluble glycoproteins has not been established conclusively [36,37]. Preliminary investigations have shown that glycoconjugate-bound Neu5Gc is present regularly in amounts lower than 0.01% of the total sialic acid fraction in human tissues and in blood plasma glycoproteins [38]. However, there are indications from studies with mice that low amounts of Neu5Gc in glycoconjugates might be of nutritional origin [39]. So far, the occurrence of *N*-acetylneuraminate mono-oxygenase (EC 1.14.99.18) activity, responsible for the conversion of CMP-Neu5Ac into CMP-Neu5Gc [37,40], has not been demonstrated to come to expression in normal adult human tissue [41].

Extensive studies have shown that when normal adult humans are exposed to sera of animal species, immunogenic responses may occur. The so-called Hanganutziu–Deicher (serum-sickness) antibodies are directed towards glycoconjugates containing terminal Neu5Gc, α2-3 linked to β-Gal [42,44]. Hanganutziu–Deicher antigens have also been detected in sera and tissues of patients suffering from various diseases including cancer, having never received sera from animals [43,45]. Literature data have indicated the occurrence of Neu5Gc in fetal human tissue [46], in certain tumors [47,48], and in various human tumor cell lines [49,50]. In case of human colon cancer, Neu5Gc-containing gangliosides were demonstrated to occur in amounts < 1% [48]. The demonstration of Neu5Gc as an oncofetal antigen in humans suggests that postnatally the expression of Neu5Gc is completely suppressed, but that re-expression of Neu5Gc can take place in certain disease states.

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