

CARBOHYDRATE FEATURES OF RECOMBINANT HUMAN GLYCOPROTEINS

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ABSTRACT

In the present approaches of developing pharmaceutically relevant drugs based on recombinant human glycoproteins, the establishment of the glycosylation patterns of these biomolecules is highly actual. In this minireview a short overview of the state of the art with respect to glycoprotein glycan analysis is given. Furthermore, the glycosylation of plasminogen activators and erythropoietins is summarized in more detail.

Key words: recombinant glycoproteins, carbohydrate analysis, plasminogen activator, erythropoietin

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RESUMEN

El establecimiento de los patrones de glicosidación de fármacos basados en glicoproteínas humanas recombinantes constituye un elemento de gran actualidad en las tendencias modernas de desarrollo de esas biomoléculas. El presente trabajo de revisión pasa revista al estado del arte del análisis de glicanos de las glicoproteínas. Además, resume en mayor detalle la glicosidación de los activadores del plasminógeno y las eritropoietinas.

Palabras claves: glicoproteínas recombinantes, análisis de carbohidratos, activador de plasminógeno, eritropoietina

Introduction

In recent years the academic and industrial interest in the Carbohydrate part of glycoproteins has grown dramatically. This is mainly due to accumulating evidence concerning the relevance of the glycan chains in the physical and biological behaviour of these biomolecules. N-linked glycoprotein glycans award essential physical properties like proper folding and stabilization of the conformation of glycoproteins, protease resistance, and charge and waterbinding capacity. Furthermore, they are relevant in biological recognition processes like protein targeting and cell-cell interactions. It has been well established that ensembles of N-linked carbohydrate chains of glycoproteins, especially the range of non-reducing terminal structural elements, are species specific, tissue specific, cell-type specific, and protein specific. The terminal sequences are often differentially expressed during early development, provide masking functions to prevent rapid clearance from circulation, activate host immunological systems, act as immunodeterminants, and influence bioactivity. The importance of O-linked carbohydrate chains has been mainly discussed in terms of charge, waterbinding properties, and stability. For comprehensive reviews, see Rademacher, *et al.* (1), Allen and Kisailus (2), Lis and Sharon (3) and Montreuil, *et al.* (4, 5).

It is without doubt that the present knowledge on the importance of glycoprotein glycosylation has strongly influenced the discussion with respect to

genetically engineered proteins prepared in heterologous cell types, with or without a glycosylation machinery (6-8). In the context of therapeutic administration of recombinant human glycoproteins, an increasing interest of pharmaceutical industries is observed to consider the glycosylation patterns of the engineered proteins, with regard to applicability, tolerance and patent position.

The development of advanced analytical methods to unravel the structures of the ensembles of carbohydrate chains, present at specific amino acid residues in polypeptide backbones, has been the first step in the glycoprotein revival, and formed the basis for what today is called "glycobiology". Although in recent years several approaches based on ¹H-NMR spectroscopy (9, 10) and mass spectrometry (11-16) have become available, and much research is focused on the development of profiling techniques based on high performance separation procedures (17-19), the primary structure analysis of glycoprotein glycans has still not reached the level of a routine analysis. Even now, completely novel oligosaccharide elements, as part of glycoprotein glycans, are identified. Therefore, it should be stressed that, due to the complexity of the carbohydrate chains, not only in terms of monosaccharide constituents and sequences, but also in terms of non-carbohydrate substituents, the current approaches have to be applied with great care.

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In this minireview, the chemical nature of glycoprotein glycans and the current methodologies for the analysis of glycosylation patterns will be shortly described. Special attention will be paid to the glycosylation of two pharmaceutically important glycoproteins, namely, human plasminogen activator and human erythropoietin.

Structural Aspects of Glycoprotein Glycans

Glycoproteins are biopolymers consisting of a polypeptide backbone with covalently attached carbohydrate chains (2-5). Two major classes occur, namely, N-glycans linked via *N*-acetyl- β -D-glucosamine (GlcNAc) to the amide nitrogen of L-asparagine (Asn), and O-glycans linked via *N*-acetyl- α -D-galactosamine (GalNAc) to the hydroxyl group of L-serine (Ser) or L-threonine (Thr). Glycoproteins may contain more than one glycosylation site, and N- as well as O-linked glycans can be attached to one polypeptide backbone. Moreover, microheterogeneity of the oligosaccharide structures attached to one amino acid residue is a known phenomenon, yielding a series of distinct glycoforms for each glycoprotein.

In general, N-glycans share a common Asn-linked pentasaccharide core element $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc}\beta 1-$ (Man, D-mannose), which can be extended in several ways. Based on typical elongations of this core element, three major types of N-glycans can be distinguished, namely, (i) the oligomannose type; (ii) the complex type, comprising the *N*-acetylglucosamine and/or the *N,N'*-diacetylglucosamine type, whereas also β -D-xylose (Xyl) attached at O2 of β Man can be present; and (iii) the hybrid type, showing characteristics of both the oligomannose and the complex type. As an illustration, in Scheme 1 two composite N-linked structures are presented, covering the major findings for (i) and (ii).

For the mentioned O-glycans, so far nine core elements can be defined, which are depicted in Scheme 2. These cores can be extended in several ways, comprising e.g. $\text{Gal}\beta 1-4\text{GlcNAc}\beta$ or $\text{Gal}\beta 1-3\text{GlcNAc}\beta$ (Gal, D-galactose) elements, and terminated by monosaccharides like L-fucose (Fuc), Gal, GalNAc or *N*-acetylneuraminic acid (Neu5Ac) / *N*-glycolylneuraminic acid (Neu5Gc) (10). Moreover, *O*-acetyl and sulfate groups can occur, as demonstrated for the N-glycans.

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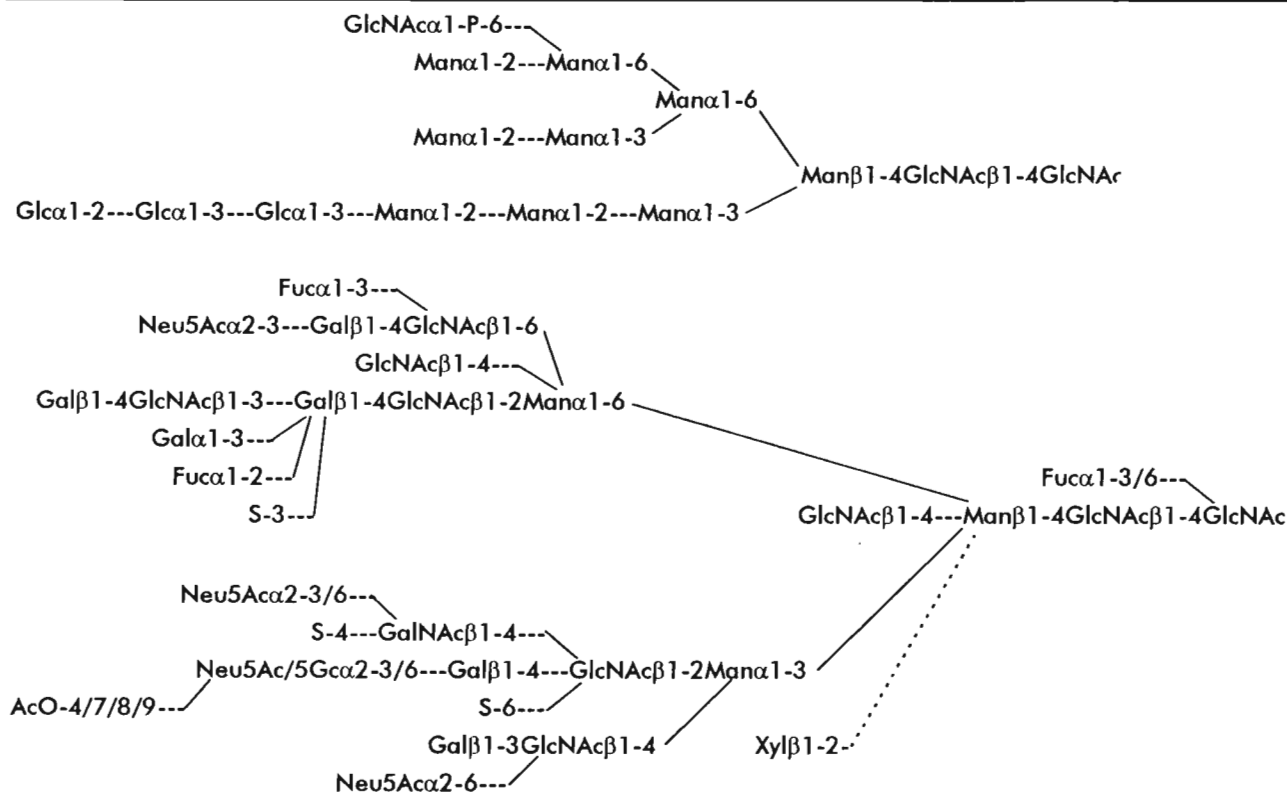
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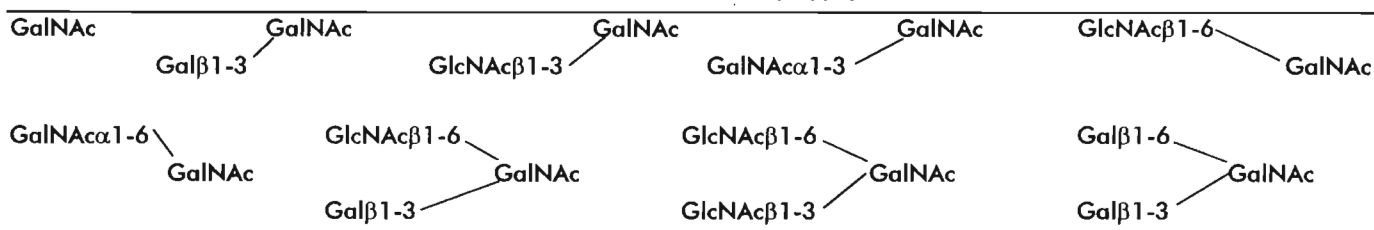
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Scheme 1. Composite N-glycan structures linked to Asn in the polypeptide via GlcNAc. Elements/units attached via ---- indicate possible chain extensions. Glc, D-glucose; Gal, D-galactose; Fuc, L-fucose; Xyl, D-xylose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; AcO, *O*-acetyl; P, phosphate; S, sulfate.



Scheme 2. Possible O-glycan core structures linked to Ser or Thr in the polypeptide via GalNAc.



In the context of this survey, it should be mentioned that in yeast glycoproteins, N-linked high-mannose chains and O-linked (to Ser or Thr) short oligomannose saccharides can occur.

Structure Analysis of Glycoprotein Glycans

In view of the presented complexity of the analytical problem, it may be evident that the determination of the primary structure of Asn-linked and of Ser- or Thr-linked carbohydrate chains on intact glycoproteins is nearly impossible. Therefore, the prepara-

tion of partial structures of the protein, having a single glycosylation site (glycopeptides), or release of the glycan chains from the glycoprotein (oligosaccharides), is a prerequisite. In Scheme 3 the major cleavage procedures for the preparation of mixtures of glycopeptides or oligosaccharides from native glycoproteins are summarized (2, 4, 5, 17-21). For a detailed analysis of the released carbohydrate chains, these mixtures have to be fractionated, and different combinations are in use. Frequently applied combinations are high-voltage paper electrophoresis and Bio-Gel P-4 gel-permeation

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Scheme 3. Major methodologies in the structure analysis of glycoprotein glycans. PNGase, peptide- N^A -(N -acetyl- β -glucosaminyI)-asparagine amidase; PA, 2-pyridylamino; LC, liquid chromatography; GLC-MS, gas-liquid chromatography / mass spectrometry; 1D, one dimensional; 2D, two dimensional; NMR, nuclear magnetic resonance.

* Isolation of glycoproteins

* Preparation of carbohydrate chains

- protease digestion → glycopeptides
- hydrazinolysis for GlcNAc-Asn type → oligosaccharides / alditols / PA derivatives
- PNGase F or A for GlcNAc-Asn type → oligosaccharides / alditols / PA derivatives
- endo-H and other endo- N -acetylglucosaminidases for GlcNAc-Asn type (mainly oligomannose type) → oligosaccharides / alditols / PA derivatives
- alkaline borohydride treatment for GalNAc-Ser/Thr type → oligosaccharide-alditols

* Fractionation and purification, if possible to homogeneity

- gel-permeation chromatography (Bio-Gel P-4) - high-voltage paper electrophoresis
- high-pH anion-exchange chromatography (CarboPac) - lectin chromatography
- high-pressure chromatography (e.g. Lichrosorb-NH₂) - fast-protein liquid chromatography (Mono Q)

* Degradation procedures

- exo-glycosidases (α -sialidases, α -D-galactosidases, β -D-galactosidases, N -acetyl- β -D-hexosaminidases, α -D-glucosidase, α -D-mannosidases, β -D-mannosidases, α -L-fucosidases, β -D-xylosidase)
- endo-glycosidases (endo- β -D-galactosidases)
- partial solvolysis
- periodate oxidation

* Monosaccharide analysis, including absolute configuration determination (GLC-MS)

* Methylation analysis (GLC-MS)

* 1D/2D NMR spectroscopy (¹H, ¹³C, ³¹P)

* Mass spectrometry (electron impact, chemical ionization, fast-atom bombardment, electrospray/ionspray, matrix-assisted laser desorption ionization)

* Profiling (LC mapping techniques, high-pH anion-exchange chromatography, capillary electrophoresis, fluorophore-assisted-carbohydrate electrophoresis, reagent-array analyses; combination with exoglycosidases or mass spectrometry)

chromatography (19, 20, 22), or Mono Q/Resource Q, Lichrosorb-NH₂ and CarboPac liquid chromatography (23, 24). For additional information, see also Fukuda and Kobata (17), Hounsell (18), and Lennarz and Hart (19). The structural analysis of isolated carbohydrate chains can be carried out along different routes. Major strategies include methylation analysis (12), exo- and endo-glycosidase studies (2, 17-20), ¹H-NMR spectroscopy (9, 10) and mass spectrometry (11, 13, 15, 16). Quite often these approaches are used in combination with each other. But also other approaches have been reported (for general reviews, see Fukuda and Kobata (17), Hounsell (18), and Lennarz and Hart (19)). At present, great effort is placed on the development of profiling procedures, which can be applied to get an impression of the carbohydrate chains present in unknown glycoproteins, or will be of use in controlling possible glycan microheterogeneity shifts in known glycoproteins (batch control). In this respect, more-dimensional HPLC mapping techniques (25), high-pH anion-exchange chromatography (26, 27), capillary electrophoresis (28, 29), fluorophore-assisted-carbohydrate electrophoresis (30), and so-called reagent-array analyses (31) seem to be highly promising.

Over the years, part of our research projects have been focused on the glycan analysis of a wide range of glycoproteins derived from many different biological sources. For an idea of our activities in this field during the last five years, see Bergwerff, *et al.* (32-35), Damm, *et al.* (36), De Waard, *et al.* (37), Gerwig, *et al.* (38), Hård, *et al.* (23, 39-41), Hokke, *et al.* (24, 42, 43), Kubelka, *et al.* (44), Pfeiffer, *et al.* (45), Van Dam, *et al.* (46) and Van Zuylen, *et al.* (47).

Recombinant Human Glycoproteins

Several reports have appeared in the literature focusing on primary structure analysis of recombinant glycoproteins and on the biological significance of the specific glycosylation patterns. Typical examples of the structure analysis of recombinant human glycoprotein glycans, as carried out in our research group, comprise the carbohydrate chains of γ -interferon (N-glycoprotein; 48), follitropin (N-glycoprotein; 39), chimeric plasminogen activator (N-glycoprotein; 32) and erythropoietin (N,O-glycoprotein; 43, 49, 50), all produced in Chinese hamster ovary (CHO) cells. Furthermore, detailed studies were carried out on the glycan chains of IgG1 antibodies (N-glycoprotein; 34), produced in murine SP2/0 transfectoma cell subclones, and of insulin-like growth factor I (O-glycoprotein; 51), produced in *Saccharomyces cerevisiae* cells. In all these cases, ¹H-NMR spectroscopy was applied as the major analytical technique.

Plasminogen Activators

Plasminogen activators (PA) are serine proteases, that catalyze the conversion of inactive plasminogen into active plasmin (52-54). Plasmin is considered to be the primary circulating fibrinolytic enzyme, which can clear thrombi from blood vessels by the degradation of the fibrin network, a process called fibrinolysis. In view of this feature, PA's are of clinical interest for the treatment of thrombolytic disorders. Two major activators have been identified, namely, tissue-type plasminogen activator (t-PA) and urinary-type plasminogen activator (u-PA). Besides the generation of recombinant plasminogen activators, hybrid variants have also been constructed, and in the framework of this minireview one of these chimeric plasminogen activators, K₂tu-PA, is included.

Tissue-Type Human Plasminogen Activators

The polypeptide backbone of human t-PA, having a M_r of about 70 kDa and built up from 527 amino acids (an N-terminal extension of 3 amino acids not included), consists of an N-terminal finger (F) domain, an epidermal growth factor (E) domain, two kringle (K₁ and K₂) domains, and a C-terminal protease (P) domain (54). It exists in a single-chain form and in a two-chain form, and the single-chain form can be converted into the two-chain form by the action of proteases like plasmin (cleavage between Arg-275 and Ile-276). In the two-chain form an A-chain comprises the domains F, E, K₁ and K₂, and a B-chain correlates with the P domain; the two polypeptides are linked through a disulfide bridge. Several reports have discussed the influence of the glycosylation of t-PA on its biochemical / biological properties, and specific information can be obtained from Hansen, *et al.* (55), Beebe and Aronson (56), Collen, *et al.* (57), Lucore, *et al.* (58), Wittwer, *et al.* (59), Wittwer and Howard (60), Howard, *et al.* (61), Otter, *et al.* (62), Berg, *et al.* (63), and Hajjar and Reynolds (64).

t-PA is N- and O-glycosylated, and has a carbohydrate content of approximately 7%. Two variants of t-PA occur, namely, type I t-PA, which is N-glycosylated at Asn-117 (K₁ domain), Asn-184 (K₂ domain), and Asn-448 (P domain), and type II t-PA, which is glycosylated at Asn-117 and Asn-448. A Fuc residue is O-linked to Thr-61 in the E domain. So far, the primary structure of the human serum t-PA N-glycans has not been determined, but studies addressing the elucidation of the glycan chains of non-recombinant t-PA from human colon fibroblast cells (65) and Bowes melanoma cells (65-67), and of recombinant human t-PA from CHO cells (49, 68-70) and C127 mouse epithelial cells (45, 69, 71) have been reported. An overview of the results is presented in Table 1. An evaluation of the various studies shows that each cell type produces t-PA with

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Table 1. Survey of structural information concerning major N-glycosylation patterns of human tissue-type plasminogen activators.

Cell Type	N-glycosylation sites		
	Asn-117	Asn-184 (+/-)	Asn-448
Human colon fibroblast cells (65)	predominantly oligomannose type	predominantly N-acetylglucosamine type; Neu5Ac in α 2-6 linkage	predominantly N-acetylglucosamine type; Neu5Ac in α 2-6 linkage
Bowes melanoma cells (67)	almost exclusively oligomannose type	predominantly N,N'-diacetylglucosaminosyl type; Neu5Ac in α 2-6 linkage	predominantly N,N'-diacetylglucosaminosyl type; Neu5Ac in α 2-6 linkage
Chinese hamster ovary cells (49, 67, 70)	almost exclusively oligomannose type	predominantly N-acetylglucosamine type; Neu5Ac in α 2-3 linkage; traces of Neu5Gc	exclusively N-acetylglucosamine type; Neu5Ac in α 2-3 linkage; traces of Neu5Gc
C127 mouse epithelial cells (45, 71)	predominantly oligomannose type	N-acetylglucosamine type; Neu5Ac in α 2-3/6 linkage; Neu5Ac α 2-3(SO ₄ -6)-Gal; Gal α 1-3Gal	N-acetylglucosamine type; Neu5Ac in α 2-3/6 linkage; Neu5Ac α 2-3(SO ₄ -6)-Gal; Gal α 1-3Gal

a unique glycosylation pattern, wherein oligomannose-, N-acetylglucosamine-, N,N'-diacetylglucosaminosyl- and/or hybrid-type structures can play a role. Of special interest are the differences in the type of linkage for sialic acid (α 2-6 versus α 2-3), the predominant/almost exclusive glycosylation of Asn-117 with oligomannose-type structures, the exclusive occurrence of N,N'-diacetylglucosaminosyl-type structures in t-PA from Bowes melanoma cells, and the exclusive sulphated structure in recombinant t-PA from C127 mouse epithelial cells (Scheme 4). In some of the studies the detection of oligo-(N-acetylglucosamine) elements was mentioned. The different studies focused on t-PA from Bowes melanoma cells clearly illustrate the complexity of glycan analysis.

Comparison of the glycan ensembles of type I and type II t-PA showed that the presence (type I) or absence (type II) of carbohydrate chains at Asn-184 did not affect the glycosylation patterns at Asn-117 or at Asn-448 (70). Furthermore, a recombinant t-PA from CHO cells bearing a single N-glycosylation site at Asn-448 (Gln in stead of Asn at positions 117 and 184) demonstrated the only presence of N-acetylglucosamine-type chains (72). The creation of new N-glycosylation sites in the E domain of recombinant t-PA from C-127 cells afforded interesting results (73). A replacement of Tyr-67 by Asn leads to an exclusively oligomannose-type glycosylation of Asn-117, and N-acetylglucosamine-type glycosylations of Asn-67, Asn-184 and Asn-448. However, a replacement of Gly-60 by Ser leads, besides N-acetylglucosamine-type glycosylations of Asn-58, Asn-184 and Asn-448, to a predominantly N-acetylglucosamine-type glycosylation of Asn-117. For additional studies with respect to alterations in the domain structures of t-PA and the effects on glycosylation patterns, see Wilhelm, *et al.* (74). Interestingly, a recent study appeared dealing with t-PA co-expressed in CHO cells with α 2-6 sialyltransferase, affording N-glycans with α 2-6 linked sialic acid (75).

Urinary-Type Human Plasminogen Activator

The polypeptide backbone of human u-PA, having a M_r of about 54 kDa and built up from 411 amino acids, consists of an N-terminal epidermal growth factor (E) domain, a kringle (K) domain, and a C-terminal protease (P) domain (54). It is mainly synthesized as a single-chain form, called pro-urokinase, but for the greater part converted into a two-chain form, called urokinase, by the action of proteases like plasmin (cleavage between Lys-158 and Ile-159). In urokinase the two polypeptides (A-chain/E-K domains and B-chain/P domain) are linked through a disulfide bridge. u-PA has gained biomedical interest, since it is also involved in physiological and pathological tissue destruction and cell migration processes, such as gland involution and tumor growth (52, 76). Only limited biochemical information focused on the glycosylation is available, and can be obtained from Zamarron, *et al.* (77), Sarubbi, *et al.* (78), Henkin, *et al.* (79), Li, *et al.* (80) and Lenich, *et al.* (81).

u-PA is O- and N-glycosylated, and has a carbohydrate content of approximately 2%. A Fuc residue is O-linked to Thr-18 in the E domain. The P domain contains an N-glycosylation site at Asn-302, and recently a study describing the analysis of eleven oligosaccharides has been completed (35, 82). Detailed information has been included in table 2. The main compound (30%) is a diantennary chain with two SO₄-4GalNAc β 1-4GlcNAc antennae. Of further interest is the GalNAc β 1-4(Fuc α 1-3)GlcNAc sequence, being a variant of the well-known Lewis x determinant.

Human Chimeric Plasminogen Activator K₂tu-PA

In the framework of developing improved PA variants, a hybrid variant of t-PA and u-PA, namely K₂tu-PA, has been constructed, wherein sequences coding for the K₂ domain of t-PA and for the P domain of u-PA, were combined on the gene level, and CHO cells were chosen as expression system (83).

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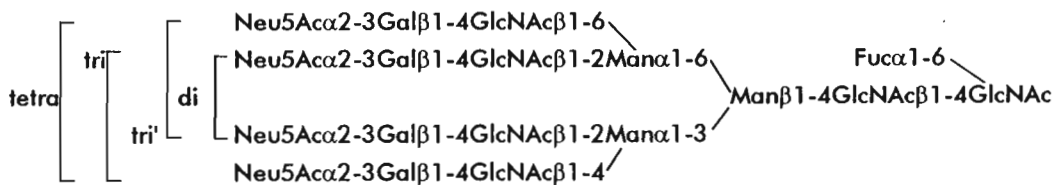
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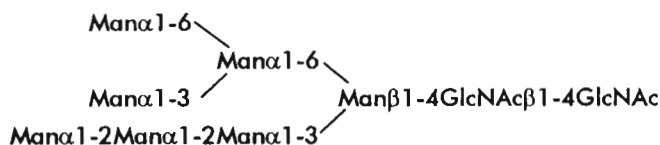
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Scheme 4. Interesting glycan structures of recombinant t-PA expressed in different cell types.

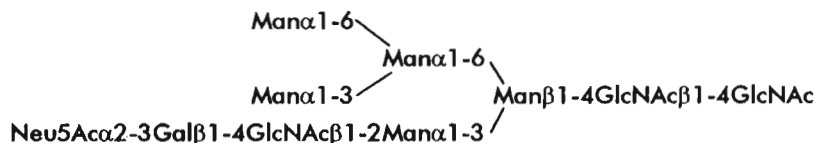
* di-, tri-, tri', and tetra-antennary sialylated N-acetylglucosamine-type chains:



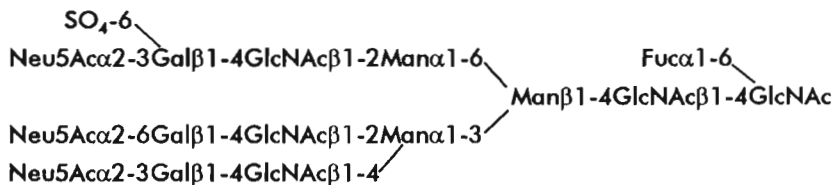
* N,N'-diacetylglucosamine-type chain in Bowes melanoma t-PA:

* oligomannose-type chains, e.g. Man₇GlcNAc₂:

* hybrid-type chain:



* sulphated tri-antennary chain in C-127 t-PA:



The chimeric PA was designed in order to combine the fibrin specificity of t-PA with the catalytic activity of u-PA. K₂tu-PA is partially glycosylated at Asn-12 (originally Asn-184 of t-PA) and completely glycosylated at Asn-247 (originally Asn-302 of u-PA), and has a carbohydrate content of approximately 6%. A plasmin cleavage site, yielding the A- and B-chains, occurs between Arg-103 and Ile-104. In table 3 the structures of the various glycan chains are presented (32). They clearly represent the glycosylation machinery of CHO cells (Scheme 5). Of interest is the finding of minor amounts of Neu5Gc and Neu5,9Ac₂ residues, besides the major sialic acid Neu5Ac. A discussion on the finding of Neu5Gc and the possible relevance of its appearance in recombinant human glycoproteins of pharmaceutical interest is reported by Hokke, *et al.* (49).

The glycosylation sites on the A- (Asn-12) and the B- (Asn-247) chain show identical arrays of microheterogeneity. On the A-chain mono- and disialylated compounds have a higher relative abundance than on the B-chain, whereas the reverse holds for the tri- and tetrasialylated compounds. It should be noted that the relative amounts of the various glycan chains in K₂tu-PA vary subtly with the applied isolation procedures.

The highly sialylated K₂tu-PA does not contain oligomannose-, hybrid-, and N,N'-diacetylglucosamine-type chains. The degree of sialylation is of interest because of clearance by the hepatic Gal (asialoglycoprotein) receptor (87). The absence of oligomannose-type structures may prolong the half-life of K₂tu-PA relative to that of t-PA, since the uptake from blood circulation by the hepatic Man re-

39. Hård K, Møkkings A, Damm JBL, Kamerling JP, De Boer W, Wijnands RA, Vliegthart JFG. Isolation and structure determination of the intact sialylated N-linked carbohydrate chains of recombinant human follitropin expressed in Chinese hamster ovary cells. *Eur J Biochem* 1990;193:263-271.

40. Hård K, Damm JBL, Spruijt MPN, Bergwerff AA, Kamerling JP, Van Dedem GWK, Vliegthart JFG. The carbohydrate chains of the β subunit of human chorionic gonadotropin produced by the choriocarcinoma cell line BeWo; novel O-linked and novel bisecting-GlcNAc-containing N-linked carbohydrates. *Eur J Biochem* 1992;205:785-798.

41. Hård K, Van Doorn JM, Thomas-Oates JE, Kamerling JP, Van Der Horst DJ. Structure of the Asn-linked oligosaccharides of apolipoprotein III from the insect *Locusta migratoria*; carbohydrate-linked 2-aminoethylphosphonate as a constituent of a glycoprotein. *Biochemistry* 1993;32:766-775.

42. Hokke CH, Roosenboom MJH, Thomas-Oates JE, Kamerling JP, Vliegthart JFG. Structure determination of the disialylated poly-(N-acetylglucosamine)-containing O-linked carbohydrate chains of equine chorionic gonadotropin. *Glycoconjugate J* 1994; 11:35-41.

43. Hokke CH, Bergwerff AA, Van Dedem GWK, Kamerling JP, Vliegthart JFG. Structural analysis of the sialylated N- and O-linked carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells; sialylation patterns and branch location of dimeric N-acetylglucosamine units. *Eur J Biochem* 1995;228:981-1008.

44. Kubelka V, Altmann F, Staudacher E, Tretter V, März L, Hård K, Kamerling JP, Vliegthart JFG. Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur J Biochem* 1993;213:1193-1204.

45. Pfeiffer G, Stimm S, Geyer R, Strube K-H, Bergwerff AA, Kamerling JP, Vliegthart JFG. Structure elucidation of sulphated oligosaccharides from recombinant human tissue plasminogen activator expressed in mouse epithelial cells. *Glycobiology* 1992;2:411-418.

46. Van Dam GJ, Bergwerff AA, Thomas-Oates JE, Rolmans JP, Kamerling JP, Vliegthart JFG, Deelder AM. The immunologically reactive O-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke *Schistosoma mansoni* have Lewis x as repeating unit. *Eur J Biochem* 1994 225:467-482.

47. Van Zuylen CWEM, De Beer T, Rademaker GJ, Haverkamp J, Thomas-Oates JE, Hård K, Kamerling JP, Vliegthart JFG. Site-specific and complete enzymic deglycosylation of the native human chorionic gonadotropin α-subunit. *Eur J Biochem* 1995; 231: 754-760.

ceptor is prohibited (62). The absence of terminal SO₄-4GalNAc (if present, proposed uptake by a hepatic SO₄-4GalNAc receptor, 88, 89) and terminal GalNAc (if present, proposed uptake by the hepatic asialoglycoprotein receptor, 90) elements may increase the half-life of K₂tu-PA relative to that of u-PA. Also the absence of O-linked Fuc in K₂tu-PA

may be of importance, since it has been shown that in t-PA this element may mediate binding and degradation by hepatoma cells, and it is suggested that this residue is involved in the uptake of the glycoprotein from the circulation (64). Finally, K₂tu-PA lacks the potential immunogenic determinant GalNAcβ1-4(Fuca1-3)GlcNAc.

Table 2. Survey of the major N-glycosylation pattern of human urinary-type plasminogen activator (35, 82).

Compound	Percentage
$\begin{array}{l} \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	30
$\begin{array}{l} \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{Fuca1-3} \\ \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \\ \text{Fuca1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \\ \text{Fuca1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{Fuca1-3} \\ \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{GalNAc} \\ \text{SO}_4\text{-4GalNAc} \end{array} \left \begin{array}{l} \text{GalNAc} \\ \text{Neu5Ac}\alpha\text{2-6GalNAc} \end{array} \right \left \begin{array}{l} \text{Neu5Ac}\alpha\text{2-3Gal} \\ \text{SO}_4\text{-4GalNAc} \end{array} \right \left\{ \begin{array}{l} \beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \right. \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-6} \\ \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7
$\begin{array}{l} \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6} \\ \text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-3} \\ \text{Neu5Ac}\alpha\text{2-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-4} \end{array} \left\{ \begin{array}{l} \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \\ \text{Man}\beta\text{1-4GlcNAc}\beta\text{1-4GlcNAc} \end{array} \right. \begin{array}{l} \text{Fuca1-6} \\ \text{Fuca1-6} \end{array}$	<7

Table 3. Survey of the major (> 95 %) N-glycosylation pattern of human chimeric plasminogen activator K₂tu-PA (32).

Compound		Percentage*
-	Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	8 35 ^{a,b}
-	Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3	1.5 20 ^{c,d}
-	Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	0.5 6 ^{e,f}
	Neu5Acα2-3Galβ1-4GlcNAcβ1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	13 ^{g,h}

^a 1x Neu5Ac + 1x Neu5Gc, 2 %.

^b 1x Neu5Ac + 1x Neu5,9Ac₂, 8 %.

^c 2x Neu5Ac + 1x Neu5Gc, amount not determined.

^d 2x Neu5Ac + 1x Neu5,9Ac₂, 1 %.

^e 2x Neu5Ac + 1x Neu5Gc, amount not determined.

^f 2x Neu5Ac + 1x Neu5,9Ac₂, 1 %.

^g 3x Neu5Ac + 1x Neu5Gc, 3 %.

Erythropoietin

Human erythropoietin (EPO), a glycoprotein that is synthesized mainly by the kidney, stimulates the proliferation and differentiation of erythroid progenitor cells (91). The polypeptide backbone of active EPO (M_r 34 kDa) is built up from 165 amino acids, and has three N-glycosylation sites at Asn-24, Asn-38, and Asn-83, respectively, and one O-glycosylation site at Ser-126. Its average carbohydrate content is about 40 % (92-94). Urinary EPO is generally isolated from pools of urine of aplastic anemic patients. EPO has been expressed in various heterologous cell systems and the recombinant human glycoprotein is an important therapeutic agent for the treatment of anemia associated with renal failure (93, 95, 96). From several studies, it has become clear that the glycosylation is of great importance for the biological functioning of EPO. It could be demonstrated that removal or modification of the glycan chains, or prevention of the glycosylation at specific sites by site-directed mutagenesis, results in altered *in vivo* and *in vitro* activity (95, 97-105).

So far, studies have mainly been reported, describing the generation of recombinant human EPO in cells having a glycosylation machinery, such as

CHO cells and baby hamster kidney (BHK) cells. With respect to the primary structure analysis of the carbohydrate chains of urinary EPO and recombinant EPO expressed in CHO and BHK cells, several investigations have been published (Urinary EPO: see 106-109; CHO EPO: see 43, 106, 108-114; BHK EPO: see 107, 115-117). It should be emphasized that the various research groups studied different batches of EPO's supplied by different companies. Also the use of other cell lines has been evaluated, and typical examples comprise ψ2 cells derived from NIH/3T3 (118), insect cells (119), and cultured tobacco cells (120).

In Table 4, the monosaccharide analysis data of urinary and CHO EPO's, reported in the literature (43, 106), are compared (see also 107). At a first glance these data are quite similar, although a tendency may exist in that CHO EPO may contain slightly higher Gal and/or GlcNAc values. However, because different ensembles of carbohydrate chains can give rise to similar monosaccharide analyses, it is dangerous to draw detailed conclusions in terms of identity, similarity or non-identity of EPO's directly from such an analysis (see below).

The most detailed study so far on the N,O-glycosylation of EPO has been reported by Hokke, *et al.*

48. Mutsaers JHGM, Kamerling JP, Devos R, Guisez Y, Fiers W, Vliegthart JFG. Structural studies of the carbohydrate chains of human γ-interferon. *Eur J Biochem* 1986; 156:651-654.

49. Hokke CH, Bergwerff AA, Van Dedem GWK, Van Oostrum J, Kamerling JP, Vliegthart JFG. Sialylated carbohydrate chains of recombinant human glycoproteins expressed in Chinese hamster ovary cells contain traces of N-glycolylneuraminic acid. *FEBS Lett* 1990;275: 9-14.

50. Hokke CH, Kamerling JP, Van Dedem GWK, Vliegthart JFG. Determination of the branch location of extra N-acetylglucosamine units in sialo N-linked tetraantennary oligosaccharides. *FEBS Lett* 1991; 286:18-24.

51. Hård K, Bitter W, Kamerling JP, Vliegthart JFG. O-Mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in *Saccharomyces cerevisiae*. *FEBS Lett* 1989;248:111-114.

52. Dane K, Andreassen PA, Grandahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation, and cancer. *Adv Cancer Res* 1985;44:139-266.

53. Wun T-C. Plasminogen activation: biochemistry, physiology, and therapeutics. *CRC Crit Rev Biotech* 1988; 8:131-148.

Scheme 5. Survey of structural aspects of Chinese hamster ovary cell derived recombinant glycoproteins.

Carbohydrate chains of recombinant human glycoproteins expressed in CHO cells

- * can contain
- (phosphorylated) oligomannose-type structures
 - hybrid-type structures
 - N-acetylglucosamine-type structures with mono-, di-, tri-, tri', and tetra-antennae, including oligo-(N-acetylglucosamine) extensions
 - terminal sialic acid (Neu5Ac, Neu5,9Ac₂ and Neu5Gc), but only in α 2-3 linkage to Gal^a
- * have not shown terminations
- with sialic acid in α 2-6 linkage to Gal^a
 - with intersecting GlcNAc (GlcNAc β 1-4Man β 1-4 element)
 - with Gal α 1-3Gal^b
 - via GalNAc β 1-4GlcNAc (N,N'-diacetylglucosaminamine type)^c

^a Neu5Ac in α 2-6 linkage has only been reported by Davidson and Castellino (84).

^b This termination has only been described by Ashford, *et al.* (85).

^c A GalNAc-transferase activity has been detected in CHO cells by Dharmesh, *et al.* (86).

(43, 49, 50) for CHO EPO. It includes the identification of over 35 different N-linked and two O-linked oligosaccharides, representing at least 95 mol/100 mol carbohydrate chains, and the results of this N- and O-glycosylation study are presented in Tables 5 and 6, respectively. Although the literature results of the various studies on the N,O-glycosylation of urinary, CHO and BHK EPO's are to some extent similar, discrepancies are observed on a more detailed level. The discrepancies hold even for probes of a similar biological origin, investigated by different research groups. It may be evident that the structural data published for EPO support the statement in the Introduction that primary structure analysis of glycoprotein glycans has still not reached the level of a routine analysis, which is also of importance for the growing interest in profiling techniques.

Comparison of the structures of the N-glycans of urinary, CHO and BHK EPO's shows qualitatively the same branching patterns (di-, tri-, tri', tetra-) for the N-acetylglucosamine-type of chains (Table 5). The major portion of the identified carbohydrate chains comprise sialylated tetraantennary oligosaccharides with 0-2 Gal β 1-4GlcNAc β 1-3 (N-acetylglucosamine) repeats. However, it should be noted that large differences have been observed between different pools/individuals when comparing the amounts of di- and tetraantennary structures for uri-

nary EPO (107, see also 106). A similar finding has been reported for specific CHO EPO probes (111). Based on the analysis of the ensembles of N-glycans in more than 30 different batches of BHK EPO, Nimtz, *et al.* (116) indicated that occasionally di- and tri/tri'antennary oligosaccharides were barely detectable. From a biological point of view, a high content of tetraantennary carbohydrate chains is important since there is a positive correlation between the *in vivo* activity of recombinant EPO and the ratio of tetra- to diantennary oligosaccharides (111). Also, a high degree of sialylation (see Table 5) is of great importance since terminal Gal residues are recognized by the hepatic Gal (asialoglycoprotein) receptor resulting in a rapid clearance of poorly sialylated EPO from the blood stream, having a negative effect on the *in vivo* activity (95).

The studies reported by Tsuda, *et al.* (107) and Takeuchi, *et al.* (108) showed a clear tendency that CHO and BHK EPO's contain a higher content of N-linked carbohydrate chains with Gal β 1-4GlcNAc β 1-3 (N-acetylglucosamine) repeats than the urinary form. In recombinant EPO's the repeating N-acetylglucosamine units were found to be, at any case, in the branches arising from the α 1-6 linked Man residue: Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-6Man α 1-6 and/or Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-2Man α 1-6

Table 4. Monosaccharide analysis data of urinary and CHO erythropoietins (43, 106). Mannose is taken as 3.0.

	Fuc	Man	Gal	GlcNAc	GalNAc	Sialic acid
Urinary EPO	0.9	3.0	4.2	5.3	0.3	3.4
CHO EPO	0.8-1.4	3.0	4.3-5.7	5.9-6.5	trace-0.5	3.1-4.3

54. Lijnen HR, Collen D. Strategies for the improvement of thrombolytic agents. *Thromb Haemostas* 1991;66: 88-110.

55. Hansen L, Blue Y, Barone K, Collen D, Larsen GR. Functional effects of asparagine-linked oligosaccharide on natural and variant human tissue-type plasminogen activator. *J Biol Chem* 1988;263:15713-15719.

56. Beebe DP, Aronson DL. Turnover of tPA in rabbits: influence of carbohydrate moieties. *Thromb Res* 1988;51:11-22.

57. Collen D, Stassen J-M, Larsen G. Pharmacokinetics and thrombolytic properties of deletion mutants of human tissue-type plasminogen activator in rabbits. *Blood* 1988;71:216-219.

58. Lucore CL, Fry ETA, Nachowiak DA, Sobel BE. Biochemical determinants of clearance of tissue-type plasminogen activator from the circulation. *Circulation* 1988; 77:906-914.

59. Wittwer AJ, Howard SC, Carr LS, Marakas NK, Feder J, Parekh RB, Rudd PM, Dwek RA, Rademacher TW. Effects of N-glycosylation on *in vitro* activity of Bowes melanoma and human colon fibroblast derived tissue plasminogen activator. *Biochemistry* 1989;28:7662-7669.

60. Wittwer AJ, Howard SC. Glycosylation at Asn-184 inhibits the conversion of single-chain to two-chain tissue-type plasminogen activator by plasmin. *Biochemistry* 1990; 29:4175-4180.

61. Howard SC, Wittwer AJ, Welply JK. Oligosaccharides at each glycosylation site make structure-dependent contributions to biological properties of human tissue plasminogen activator. *Glycobiology* 1991;1:411-417.

62. Otter M, Barrett-Bergshoeff MM, Rijken DC. Binding of tissue-type plasminogen activator by the mannose receptor. *J Biol Chem* 1991;266:13931-13935.

63. Berg DT, Burck PJ, Berg DH, Grinnell BW. Kringle glycosylation in a modified human tissue plasminogen activator improves functional properties. *Blood* 1993;81:1312-1322.

64. Hajjar KA, Reynolds CM. α -Fucose-mediated binding and degradation of tissue-type plasminogen activator by HepG2 cells. *J Clin Invest* 1994;93:703-710.

65. Parekh RB, Dwek RA, Thomas JR, Opednakker G, Rademacher TW, Wittwer AJ, Howard SC, Nelson R, Siegel NR, Jennings MG, Marakas NK, Feder J. Cell-type-specific and site-specific N-glycosylation of type I and type II human tissue plasminogen activator. *Biochemistry* 1989;28:7644-7662.

66. Pohl G, Kenne L, Nilsson B, Einarsson M. Isolation and characterization of three different carbohydrate chains from melanoma tissue plasminogen activator. *Eur J Biochem* 1987; 170:69-75.

Table 5. Conti.

Compound		Percentage			
Neu5Acα2-3 Neu5Acα2-3	Neu5Acα2-3 -	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	1	3	19 ^{b,c}
Neu5Acα2-3 -	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3Galβ1-4GlcNAcβ1-3 -	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	2	1	
Neu5Acα2-3 Neu5Acα2-3	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3 Galβ1-4GlcNAcβ1-3	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	1	5 ^d	
Neu5Acα2-3 Neu5Acα2-3	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	5 ^e	<1	
Neu5Acα2-3 Neu5Acα2-3	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	<1	7	
Neu5Acα2-3 -	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc	<1		
Neu5Acα2-3 -	Neu5Acα2-3 Neu5Acα2-3	Galβ1-4GlcNAcβ1-2Manα1-3 Galβ1-4GlcNAcβ1-4			
Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4	Neu5Acα2-3Galβ1-4GlcNAcβ1-3 Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Neu5Acα2-3Galβ1-4GlcNAcβ1-4				

^d 2x Neu5Ac + 1x Neu5,9Ac2, <1 %.

^b 3x Neu5Ac + 1x Neu5,9Ac2, 2 %.

^c 3x Neu5Ac + 1x Neu5Gc, 2 % +

^d 3x Neu5Ac + 1x Neu5Gc, <1 %.

^e 3x Neu5Ac + 1x Neu5Gc, <1 %.

Table 6. Survey of identified O-glycosylation patterns of urinary and recombinant erythropoietin samples.

Structure	Urinary EPO		CHO EPO					BHK EPO
	Sasaki, <i>et al.</i> (106)	Inoue, <i>et al.</i> (109)	Sasaki, <i>et al.</i> (106)	Inoue, <i>et al.</i> (109)	Linsley, <i>et al.</i> (114)	Watson, <i>et al.</i> (113)	Hokke, <i>et al.</i> (43)	Nimtz, <i>et al.</i> (116)
	Relative amounts (mol %)							
GalNAc	-	70	-	-	+	-	-	-
Galβ1-3 GalNAc	-	-	-	-	+	-	-	-
Neu5Acα2-6 GalNAc	-	30	-	-	+	-	-	-
Neu5Acα2-6 Galβ1-3 GalNAc	-	-	-	32	-	-	-	-
Neu5Acα2-3Galβ1-3 GalNAc	-	-	+	32	+	+	40	70
Neu5Acα2-6 Galβ1-3 GalNAc	+	-	++	36	+	+	60	30
Neu5Ac ₃ HexHexNAc	-	-	+	-	-	-	-	-

(the *only* extensions reported by Hokke, *et al.* (43), Takeuchi, *et al.* (108), Rice, *et al.* (112)). For a survey of the *N*-acetylglucosamine extensions, as found by Hokke, *et al.* (43), see Table 5.

Focusing on tetraantennary glycans of CHO EPO also the sequence Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-4Manα1-3 has been identified (106, 113). Small amounts of tetraantennary carbohydrate chains with three repeats have been reported in Sasaki, *et al.* (106), Watson, *et al.* (113), and Linsley, *et al.* (114). In this case the third extension can comprise the Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-4Manα1-3 element, but also the Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3 sequence has been identified (106). Also the occurrence of trimeric *N*-acetylglucosamine units has been mentioned to occur (106). Focusing on tetraantennary glycans of BHK EPO, also extensions with Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-2Manα1-3 have been established (107, 116). The presence of repeats in di- and triantennary glycans of CHO EPO has been described by Hokke, *et al.* (43), and the presence of a repeat, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-4Manα1-3, in triantennary glycans of CHO EPO by Sasaki, *et al.* (106) (see also 116). Linsley, *et al.* (114) reported the existence of repeats for both the tri- and the triantennary structures. For urinary and BHK EPO, the occurrence of a Galβ1-3GlcNAcβ1-4Manα1-3 instead of a Galβ1-4GlcNAcβ1-4Manα1-3 sequence in tri- and tetraantennary glycans has been proposed by Tsuda, *et al.* (107). In a few BHK EPO batches small amounts of

N,N'-diacetylglucosamine or hybrid type of chains were observed (116).

In all studies focused on CHO EPO's the linkage between sialic acid and galactose in the N-glycans was demonstrated to be exclusively α2-3 (Table 5), whereby Hokke, *et al.* (43, 49) showed that besides Neu5Ac as the major sialic acid (95 %) also Neu5Gc (2 %) and Neu5,9Ac₂ (3 %) do occur. Linsley, *et al.* (114) described for each detected Neu5Ac-containing compound a satellite wherein one Neu5Ac residue was replaced by one Neu5Gc residue (see also Table 5). The same type of linkage with Neu5Ac was also established for BHK EPO's, and in this case Neu5Gc has been mentioned as a possible minor sialic acid constituent (116). Inconsistencies exist with respect to the type of linkage in urinary EPO glycan chains. Based on methylation analysis data, Sasaki, *et al.* (106) found evidence for the exclusive existence of α2-3 linkages, whereas Takeuchi, *et al.* (108) demonstrated the occurrence of both α2-3 (major) and α2-6 (occasionally) linkages.

The occurrence of sulphate groups in urinary and CHO EPO has been suggested (108, 121), but structural studies have not been published thus far. With respect to the α1-6 fucosylation of the Asn-bound GlcNAc residues in CHO and BHK EPO's, amounts between at least 80 % (106, 108, 113) and nearly 100 % (43, 116) have been reported. In BHK EPO the presence of a mannose-6-phosphate group has been demonstrated (117).

In Table 6 a survey of identified O-glycosylation patterns of urinary, CHO and BHK EPO's is pre-

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sented. Comparing the results of the seven studies on recombinant EPO's (43, 106, 109, 113-116) it is clear that the major carbohydrate chains are the trisaccharide Neu5Ac α 2-3Gal β 1-3GalNAc and the tetrasaccharide Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)-GalNAc. Additional O-linked chains comprise in general desialylated forms of these oligosaccharides. Interestingly, Linsley, *et al.* (114) also reported the disaccharide Neu5Ac α 2-6GalNAc and its desialylated form, the same saccharides as found by Inoue, *et al.* (109) in urinary EPO, and claimed to be unique for the urinary EPO. In the case of BHK EPO, Nimtz, *et al.* (116) published an O-glycosylation percentage of only 60%. A similar finding of partial O-glycosylation was also reported for CHO EPO by Linsley, *et al.* (114).

The variations observed in the degree of sialylation, as found in the literature for recombinant EPO's, may have been caused by the use of different purification methods to obtain recombinant EPO (distribution of glycoforms which make up the final product). Other causes for the variation in the degree of sialylation and fucosylation could be the use of various cell clones, different culturing conditions, which can lead to differences in the activities of the α 2-3 sialyltransferase or α 1-6 fucosyltransferase, and the presence of sialidase activity for CHO cells recently reported in the culture fluid (122). In principle, the inconsistencies in sialylation may also have been caused partly by the use of different methods for the release and the purification of the carbohydrate chains. It should be noted that the non-random distribution of sialic acids in tetra- and diantennary glycans, as found by Hokke, *et al.* (43), is in good agreement with the branch specificity of human placental α 2-3 sialyltransferase (123). With respect to the differences observed in the relative amounts of triantennary and triantennary oligosaccharides in various recombinant probes, it should be

stated that these different ratios cannot be artefacts caused by analytical problems. They may be explained by differences in the activities of the involved glycosyltransferases for the various cell lines and culture conditions.

Finally, the site-specific N-glycosylation in CHO EPO has been studied by Sasaki, *et al.* (110) and Linsley, *et al.* (114). Di-, tri/tri'- and tetraantennary (major) glycans were detected for Asn-24, whereas Asn-38 and Asn-83 contained mainly tetraantennary chains. With respect to the site-specific N-glycosylation pattern in BHK EPO, it was established that Asn-24 was glycosylated with di-, tri/tri'- and tetraantennary chains, and hybrid-type chains, whereas Asn-38 and Asn-83 contained almost exclusively the tetraantennary chains (116). In addition, recently, a minor phosphorylated oligomannose-type carbohydrate chain, GlcNAc α 1-P-6Man α 1-2Man α 1-3[Man α 1-6(Man α 1-3)Man α 1-6]Man β 1-4GlcNAc β 1-4GlcNAc, was found at Asn-24 (117).

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