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Construction and characterization of stably transfected BHK-21 cells with human-type sialylation characteristic

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Abstract

The human Golgi enzyme CMP-NeuAc:Gal(β1–4)GlcNAc-R α2,6-sialyltransferase (ST6N) was stably coexpressed with human erythropoietin (EPO) from a BHK-21A cell line. The cell line was characterized with respect to the expression and *in vitro* activity of the ST6N and the endogenous α2,3-sialyltransferase. Detailed structural analysis of the *N*-linked carbohydrates of the rhuEPO expressed from the new cell line was performed by HPAE-PAD-mapping, MALDI/TOF-MS and methylation analysis after purification of the recombinant protein by immunoaffinity chromatography. This is the first report describing that the human α2,6-sialyltransferase is capable of sialylating, apart from Gal(β1–4)GlcNAc-R, also GalNAc(β1–4)GlcNAc-R motifs *in vivo*, which is not the case for the endogenous BHK-cell α2,3-sialyltransferase.

Abbreviations: BHK – Baby hamster kidney; CHO – Chinese hamster ovary; CMV – Cytomegalovirus; DME – Dulbecco's modified Eagle's medium; EPO – Erythropoietin; FCS – Fetal calf serum; Gal – Galactose; GalNAc – *N*-acetyl galactosamine; GlcNAc – *N*-acetyl glucosamine; HPAE-PAD – High-performance anion-exchange chromatography with pulsed amperometric detection; MALDI/TOF-MS – Matrix-assisted laser desorption/ionization time of flight mass spectrometry; Mes – 4-morpholinoethanesulfonic acid; NeuAc – *N*-acetyl neuraminic acid; PAGE – Polyacrylamide gel electrophoresis; PNGase F – Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; ST6N – CMP-NeuAc:Gal(β1–4)GlcNAc-R α2,6-sialyltransferase (EC 2.4.99.1); SV40 – Simian virus 40.

Introduction

A characteristic structural feature of human serum glycoproteins is the predominance of N-glycans terminating with NeuAc in α2,6-linkage to Gal(β1–4)GlcNAc motifs. In contrast, when produced from BHK or CHO cells, recombinant glycoproteins have NeuAc attached exclusively in α2,3-linkage to Gal residues because these host cells do not express the specific sialyltransferase that transfers NeuAc in α2,6-linkage to galactose (Conradt et al., 1987; Lee et al., 1989: Nimtz et al., 1993). Such differences in glycan structures between recombinant proteins and their natural human counterparts might be of importance for glycoproteins destined for clinal application since polypeptide linked oligosaccharides are involved in numerous important biological phenomena, e.g. inflammatory processes, antigenicity, *in vivo* targeting and circulatory half-life of the glycoproteins. In the case of human EPO the significance of NeuAc on the biological activity *in vivo* has been investigated extensively (Goldwasser et al., 1974; Goto et al., 1988). The glycoprotein hormone EPO stimulates the proliferation and differentiation of erythroid progenitor cells into mature erythrocytes and the recombinant protein is in clinical use for the treatment of anemia due to renal failure. The *in vivo* bioactivity of EPO is completely abolished when NeuAc is absent. It has been shown that terminal

NeuAc protects the hormone against clearance from circulation by the hepatic asialoglycoprotein receptor (Spivak and Hogans, 1989).

We have previously previously reported that N-glycans of glycoproteins synthesized by BHK-21A cells bear high amounts of terminal GalNac(β1–4)GlcNAc-R structures (Grabenhorst et al., 1995). *In vivo* use of glycoproteins produced from this cell line would be rapidly removed from circulation because the endogenous α2,3-sialyltransferase is unable to act on terminal GalNAc residues resulting in a low sialylation state of the oligosaccharides in glycoproteins produced thereof. The substitution of GlcNAc by β1,4-linked GalNAc in place of the β1,4-Gal in the lactosamine-type oligosaccharides has been reported in human glycoprotein synthesized by kidney epithelial cells (Bergwerff et al., 1995).

In the present paper we report on the genetic engineering of BHK-21A cells expressing human EPO aiming at the improvement of their glycosylation characteristics by transfection of the cells with a plasmid encoding the human α2,6-sialyltransferase. The *in vitro* specificity of the rat liver α2,6-sialyltransferase and the bovine colostrum α2,6-sialyltransferase towards terminal GalNAc-residues in N-glycans has been reported previously (Hokke et al., 1994; Nemansky and Van den Eijnden, 1992). Furthermore, reports published on sialylated GalNAc-motifs have identified exclusively NeuAc α2,6-linked to this sugar (see Bergwerff et al., 1995; Weisshaar et al., 1991). The stable expression of the recombinant proteins in BHK-21 cells is reported as well as the detailed description of the glycosylated recombinant EPO secreted thereof.

Material and methods

Materials

Vibrio cholerae sialidase was from Calbiochem, *Newcastle Disease Virus* sialidase was purchased from Boehringer Mannheim. Puromycin dihydrochloride was bought from Sigma. CMP-[^{14}C]NeuAc was from Amersham (Braunschweig, Germany) and was diluted with unlabelled CMP-NeuAc to the desired specific activity. The MAK 89-146/0056 was a gift from Dr. G. Zettlmeissl (Behringwerke AG Marburg, Germany). The Gal(β1–4)GlcNAcβ-O-$(CH_2)_8CO_2$Me acceptor was a gift from Dr. O. Hindsgaul (University of Alberta, Canada). The medium DME was prepared using Dulbecco's modified Eagle medium (purchased from Gibco-BRL) that was supplemented prior to use with 10 mM Hepes, 45 mM $NaHCO_3$, 2 zmM glutamine, 0.061 g L^{-1} ampicillin and 0.1 g L^{-1} streptomycin sulfate and adjusted to pH 7.1. For routinely use in cell culture 10% FCS (Sigma) were added to DME.

Cells and plasmids

A BHK-21A cell line expressing human EPO (EPO40) was obtained from Behringwerke AG, Marburg, Germany (Fibi et al., 1995). The eukaryotic expression vector pABSial (European Patent Application EP 91 11 5282) containing the entire coding sequence for human ST6N (Grundmann et al., 1990) was obtained from Dr. Zettlmeissl, Behringwerke AG, Marburg, Germany. The vector pSV2pac (Vara et al., 1986) contains a puromycin acetyltransferase cDNA under control of the SV40 promoter and confers puromycin resistance to cells harboring the vector. The cDNA sequence of a α2,3-sialyltransferase homologous to human ST3Gal III was cloned from BHK-21 cells in our laboratory (Grabenhorst et al., unpublished results).

Transfection

EPO40 cells were stable cotransfected with the plasmids pABSial and pSV2pac in a molar ratio of 4:1 or 10:1 according to the method of Graham and Van der Eb (1973). Transfection was performed in 6-well plates using exponentially growing adherent cells in 2.5 ml DME by adding 0.25 ml of the transfection solution, containing 5 μg pABSial and 1.25 μg pSV2pac (4:1) or 12.5 μg pABSial and 1.25 μg pSV2pac (10:1), respectively. Following a medium exchange 24 h post transfection cells were grown in nonselective medium for further 24 h. Afterwards, puromycin-resistant cells were selected using several medium exchanges with DME containing 5 μg ml^{-1} puromycin dihydrochloride during a time period of 3–4 weeks.

Northern blot analysis

Total RNA was isolated from EPO40 cells by the method of Chomczynski and Sacchi (1987). Samples of 20 μg or 40 μg total RNA were sized on an agarose denaturing gel and transferred onto nylon filters (GeneScreen^{TM}, DuPont). The filters were hybridized overnight with [^{32}]labeled cDNA probes that were prepared by random priming (Rediprime DNA labeling system, Amersham, Braunschweig,

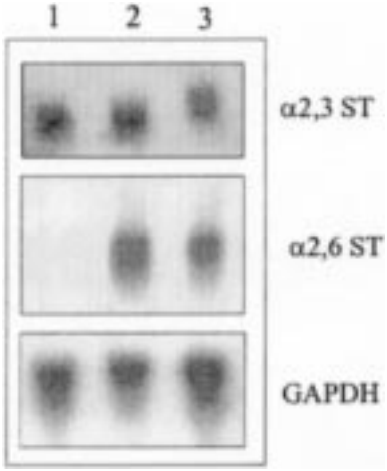

Figure 1. Northern analysis of ST6N and endogenous α2,3-sialyltransferase. Total cellular RNA was sized on an agarose gel and blotted onto a nylon membrane prior to probing with radiolabeled cDNA fragments. *Lane 1,* RNA from wild-type EPO40 cells; *lane 2 and 3,* RNA from ST6N transfected EPO-ST6NI and EPO-ST6NII cell lines, respectively. For EPO-ST6NI and EPO-ST6NII a strong hybridization signal was obtained when using probes specific for ST6N (20 μg of total cellular RNA; exposure time was 16 hours). As expected, RNA from wild-type cells failed to hybridize to the ST6N-specific probe. The mRNA of the endogenous α2,3-sialytransferase was detectable in total RNA from all cell lines, however an increased amount of RNA (40 μg) and a prolonged exposure time of up to 7 days were required. As a control BHK GAPDH was hybridized using a corresponding probe.

Germany) of a 699 bp (ST6N) and a 775 bp (ST3Gal III) PCR fragment. As a control filters were hybridized with a labeled GAPDH probe. After incubation the filters were washed under stringent conditions (Sambrook et al., 1989) and exposed to Kodak X-omat films at –70 °C for autoradiography.

Determination of cellular sialyltransferase activity

Cells from 25 cm^2 T-flasks were harvested, washed with PBS, spun at 1000 × g and resuspended at a concentration of 5×10^7 cells ml^{-1} in ice-cold extraction buffer (10 mM Mes/NaOH, pH 6.5, 2% Triton CF-54, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin) and stored at –70 °C until use. Prior to analysis the samples were thawed and incubated for 1 h on ice.

Sialytransferase activity was tested with an 8-methoxycarbonyloctyl glycoside type II acceptor substrate (Gal(β1–4)GlcNAcβ-O-$(CH_2)_8CO_2$Me) in reaction mixtures containing in a volume of 100 μl: 50 mM Mes/NaOH buffer, pH 6.5, 27 μl cell extract, 90 nmol acceptor, 5 nmol of CMP-[^{14}C]NeuAc (60.000 cpm $nmol^{-1}$), 20 mM $MnCl_2$, 100 mM NaCl and 0.2 nmol ATP. The mixture was incubated for 5 h at 37 °C, diluted to 1 ml with ice-cold H_2O and applied to Sep-Pak C_{18} cartridges. After washing with 15 ml water the products were eluted with 1.5 ml of methanol. The eluat was dried and resuspended in 700 μl sialidase buffer (50 mM Na-acetate pH 5.0, 5 mM $CaCl_2$, 0.02% NaN_3). Aliquots of 200 μl were incubated for 3 h at 37 °C with i), 10 mU *Vibrio cholerae* sialidase (releases α2,6- and α2,3-linked sialic acid), ii), 5 mU *Newcastle Disease Virus* sialidase (releases only α2,3-linked sialic acid) and iii), without sialidase. Subsequently, the reactions were stopped by the addition of water and the mixtures were applied to the Sep-Pak cartridges as described. After elution with 1.5 ml of methanol the incorporation of [^{14}C]NeuAc onto the acceptor was determined by liquid scintillation counting.

Production and purification of recombinant human EPO

Recombinant human EPO (rhuEPO) was produced for up to 3 weeks from confluently growing EPO40 cells in 175 cm^2 T-flasks in serum-free DME for 24 h followed by a 24 h period with DME containing 10% FCS. The purification of rhuEPO was achieved by immunoaffinity chromatography of the supernatant using the monoclonal anti-human EPO antibody MAK 89–146/0056 (Fibi et al., 1995) coupled to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany). The production of rhuEPO was monitored by Western blotting of the supernatants and the purity of the final preparation was verified by SDS/SAGE as detailed below.

SDS-PAGE and Western blotting

SDS-PAGE was carried out on 15% polyacrylamide gels with 3% stacking gels using the Laemmli buffer system (Laemmli, 1970). Proteins were visualized by Coomassie blue staining. For Western blot analysis, the proteins were transferred to nitrocellulose (Millipore) in a semidry instrument (Bio-Rad). The membrane was blocked with Tris-buffered saline containing 10% horse serum and 3% BSA for 1 hour and incubated overnight with a rabbit anti-EPO antiserum (Fibi et al., 1995) in blocking buffer at 1:1000 dilution. The second antibody, anti-rabbit immunoglobulin coupled to horseradish peroxidase (Nordic Immunological Laboratories, Tilburg, The Netherlands), was used at a 1:1000 dilution. The blots were developed with Tris-buffered saline containing 0.5 mg ml^{-1}

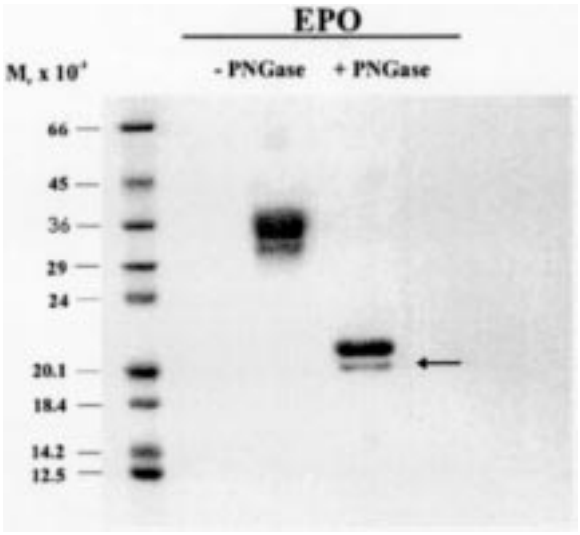

Figure 2. SDS-PAGE analysis of purified rhuEPO before and after digestion with PNGase F. 5 μg of purified EPO protein before and after enzymatic release of the N-glycans by PNGase F was dissolved in SDS sample buffer and was analyzed by SDS-PAGE using a 15% polyacrylamide gel. Proteins were stained by Coomassie brillant blue. *Lane 1*, EPO protein before incubation with PNGase F; *Lane 2*, after complete de-N-glycosylation with PNGase F. The arrow indicates the migration position of rhuEPO protein without O-linked carbohydrate; left margin, migration position of molecular mass standard proteins.

4-chloro-1-naphtol solubilized in methanol and 0.2% perhydrol.

Enzymatic release of N-glycans bound to rhuEPO

For liberation of EPO N-glycans, the purified protein was incubated with polypeptide: N-glycosidase F (PNGase) as described (Nimtz et al., 1993). Complete release of all N-linked oligosaccharides from the protein was verified by SDS/PAGE analysis. After addition of 4 vol. of –20 °C ethanol, the liberated N-glycans were recovered in the supernatant.

Analytical HPAE-PAD of oligosaccharides

A Dionex BioLC System (Dionex, Sunnyvale CA, USA) equipped with a CarboPac PA1 column (4 mm × 250 mm) was used in combination with a pulsed amperometric detector as described previously (Costa et al., 1997). Prior to HPAE-PAD analysis, the N-glycans were desialylated with Vibrio cholerae sialidase as described (Grabenhorst et al., 1995). Following desalting, the oligosaccharide material was separated by applying a 2 min isocratic run with 100% solvent A (0.2 M NaOH) followed by a linear gradient

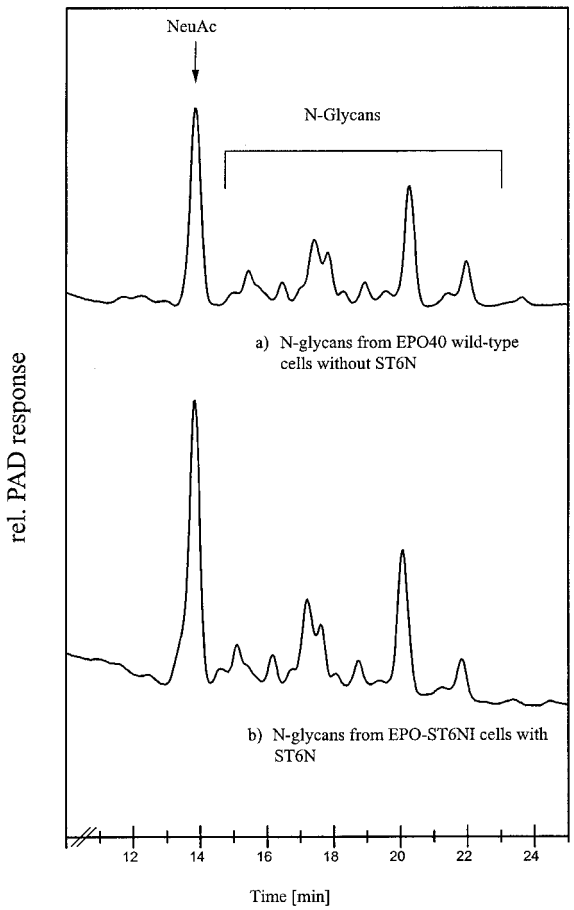

Figure 3. Analytical HPAE-PAD of desialylated rhuEPO N-glycans. Aliquots of the rhuEPO N-glycans from EPO40 wild-type cells and EPO-ST6NI cells were treated with *Vibrio cholerae* sialidase and were subjected to HPAE-PAD as described under materials and methods. The elution positions of liberated NeuAc and the neutral oligosaccharides are indicated.

of 0–20% solvent B (0.6 M sodium acetate in solvent A) over 38 min and a linear gradient to 100% B within 10 min. The flow rate was 1 ml min^{-1}.

Methylation analysis

For methylation analysis N-glycans were reduced, permethylated and purified on a Sephadex LH20 column, hydrolyzed, reduced and peracetylated according to Hakomori (1964). The separation and identification of partially methylated alditol acetates was performed on a Carlo Erba gas chromatograph, connected to a Kratos MS-50 fast-scan mass spectrometer (Nimtz et al., 1990).

Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS)

2,5-Dihydroxbenzoic acid (DHB) was used as UV-absorbing matrix. 10 mg ml^{-1} DHB were dissolved in 10% ethanol in water. For analysis by MALDT/TOF-MS the solution of the oligosaccharides was mixed with the same volume of matrix. 1 μl of the sample was spotted on a stainless steel probe tip and dried at room temperature. The concentration of the analyte was approximately 10 pmol μl^{-1}.

Measurements were performed on a Bruker REFLEX MALDI/TOF mass spectrometer using a N_2 laser (337 nm) with 3 ns pulse width and 107 to 108 W cm^{-2} irradiance at the surface (0.2 mm^2 spot). Spectra were recorded at an acceleration voltage of 20 kV using the reflectron for enhanced resolution.

Results and discussion

Consruction and isolation of BHK-21A cells stably transfected with human α2,6-sialyltransferase

The BHK-21A cell line EPO40 was cotransfected with the plasmid pABSial containing the entire coding sequence for human α2,6-sialyltransferase (ST6N) and the plasmid pSV2pac at a molar ratio of 10:1 and 4:1. Pools of stable cells were selected in the presence of 5 μg ml^{-1} puromycin hydrochloride in DME containing 10% FCS. Resistant cell clones were isolated and were assayed for expression of the newly introduced ST6N by Northern blotting. As shown in Figure 1, both, the EPO-ST6NI and EPO-ST6NII cell lines resulting from different ratios of the plasmids pABSial and pSV2pac used for transfection contained mRNA resulting from the transfected human α2,6-sialyltransferase gene that was not detected in total RNA from wild-type EPO40 cells. EPO-ST6NI cells exhibited a roughly twofold stronger signal than it was observed for EPO-ST6NII cells. The transcripts were detected using 20 μg of total cellular RNA and an exposure time of 16 h. Specific transcripts for the endogenous ST3Gal III gene were detectable in all RNA samples analyzed without significant variations in their signal intensity (see Figure 1). However, in contrast to ST6N, transcripts of ST3Gal III were only detectable when using high amounts of RNA (40 μg) and a prolonged exposure time of up to 7 days. These results indicate a significantly higher transcription level for newly introduced ST6N when compared to that of the endogenous α2,3-sialyltransferase in both, EPO-ST6NI and EPO-ST6NII cells.

Determination of sialyltransferase activity in cellular extracts

Extracts of wild-type EPO40 and transfected EPO-ST6NI cells were assayed for sialyltransferase activity with Gal(β1–4)GlcNAcβ-O-$(CH_2)_8CO_2$Me as an acceptor and CMP-[^{14}C]NeuAc as a donor substrate. Increased incorporation of radioactivity into the acceptor substrate was observed in assays employing extracts from the ST6N-transfected cells (0.25 μU of sialyltransferase/10^6 cells) when compared to extracts prepared from wild-type cells (0.06 μU of sialyltransferase/10^6 cells). This difference is most likely attributable to the newly introduced ST6N into the EPO-ST6NI cell line. To evaluate if the latter cell line indeed expresses α2,6-sialyltransferase activity, aliquots of the purified radiolabeled acceptor substrates were incubated with *Newcastle Disease Virus* (NDV) sialidase, which specifically removes α2,3-linked NeuAc only, and with *Vibrio cholerae* sialidase which removes both, the α2,3- and the α2,6-linked NeuAc. Following incubation with cellular extracts derived from EPO40 wild-type cells, the radioactivity incorporated onto the acceptor substrate could almost completely removed by the NDV sialidase and by *V. cholerae* sialidase. In contrast, more than 80% of NeuAc transferred by EPO-ST6NI cellular extracts was resistant to NDV-sialidase indicating that large amounts of NeuAc has been transferred in α2,6-linkage by the newly introduced ST6N. The amount of α2,3-linked NeuAc contributed by the endogenous α2,3-sialyltransferase of BHK cells was similar in both cell lines investigated.

Characterization of the N-linked oligosaccharides of secreted rhuEPO

In order to investigate the glycosylation characteristics of EPO-ST6NI cells, rhuEPO was produced from EPO-ST6NI cells in T-flasks from confluently growing monolayers. As a control, rhuEPO was produced from the EPO40 parent cell line. In each case about 0.8 mg rhuEPO protein was purified from 1L of culture supernatant by using immunoaffinity chromatography (Fibi *et al.*, 1995). Based on Western analysis the final yield after purification of rhuEPO was higher than 90%.

The purified protein was completely de-N-glycosylated by PNGase F and total liberated gly-

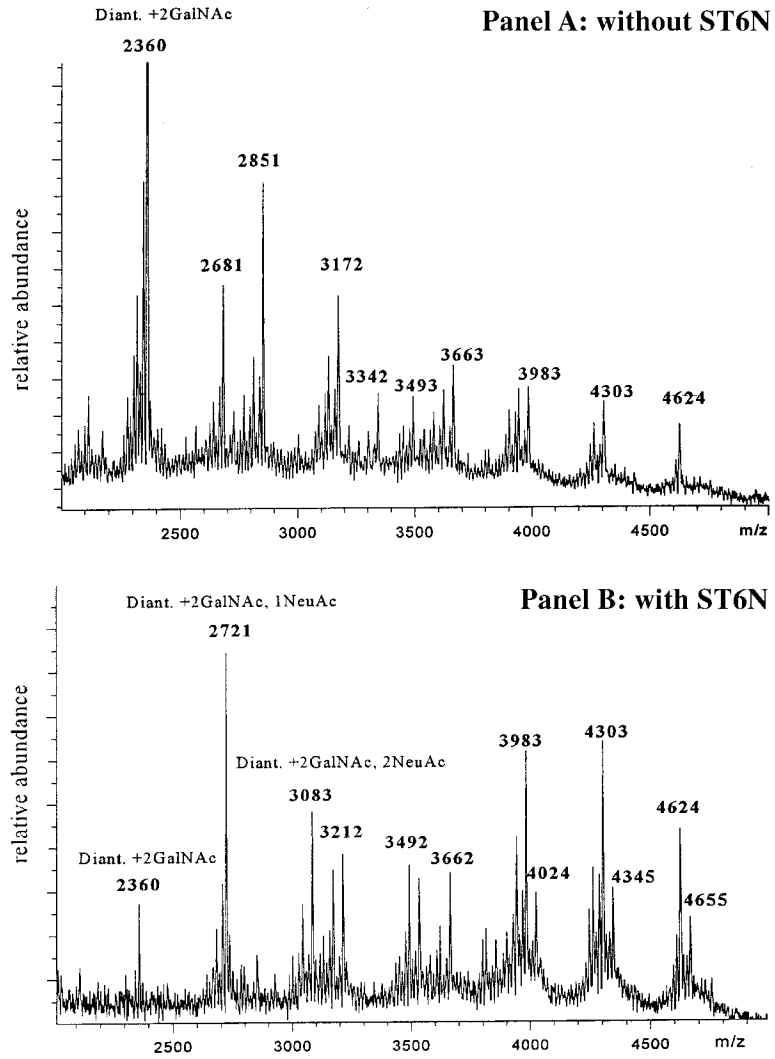

Figure 4. MALDI/TOF-MS of the reduced and permethylated total N-glycans from rhuEPO produced from BHK-21A cells with and without human ST6N. Complex-type N-glycans structures given were calculated based on the observed molecular ion signals [m/z = $(m+K)^+$] detected and on methylation analysis data (not shown). All structures were proximally fucosylated with α1-6-linked fucose.

Panel A: Positive ion MALDI/TOF mass spectra of reduced and permethylated total EPO N-glycans from EPO40 wild-type cells: 2319 Da: diantennary asialo structure (–Gal, + GalNAc); 2360 Da: diantennary asialo structure (–2 Gal + 2GalNAc); 2640 Da: diantennary monosialo structure; 2681 Da: diantennary monosialo structure (– Gal + GalNAc); 2769 Da: triantennary asialo structure (– Gal + GalNAc); 2810 Da: triantennary asialo structure (– Gal + 2GalNAc); 2851 Da: triantennary asialo structure (– 3Gal + GalNAc); 3090 Da: triantennary monosialo structure; 3130 Da: triantennary monosialo structure (– Gal + GalNAc); 3172 Da: triantennary monosialo structure (– 2 Gal + 2GalNac); 3301 Da: tetraantennary asialo structure (– 3Gal + 3GalNAc); 3172 Da: triantennary monosialo structure (– 2Gal + 2GalNac); 3301 Da: tetraantennary asialo structure (– 3Gal + 3GalNAc); 3342 Da: tetraantennary tetraantennary asialo structure (– 4Gal + 4GalNAc); 3493 Da: triantennary disialo structure (– Gal + GalNAc); 3581 Da: tetraantennary monosialo structure (– Gal + GalNAc); 3622 Da: tetraantennary monosialo structure (– 2Gal + 2GalNAc); 3663 Da: tetraantennary monosialo structure (– 3Gal + 3GalNAc); 3901 Da: tetraantennary disialo structure; 3942 Da: tetraantennary disialo structure (– Gal + GalNAc); 3983 Da: tetraantennary disialo structure (–2Gal + 2GalNAc); 4262 Da: tetraantennary trisialo structure; 4304 Da: tetraantennary trisialo structure (– Gal + GalNAc); 4624 Da: tetraantennary tetrasialo structure.

Panel B: Spectrum of total EPO N-glycans produced from transfected EPO-ST6NI cells: 2360 Da: diantennary asialo structure (– 2Gal + 2GalNAc); 2680 Da: diantennary monosialo structure (– Gal + GalNAc); 2721 Da: diantennary monosialo structure (– 2Gal + 2GalNAc); 3001 Da: diantennary structure; 3042 Da: diantennary disialo structure (– Gal + GalNAc); 3083 Da: diantennary disialo structure (– 2Gal + 2GalNAc); 3130 Da: triantennary monosialo structure (– Gal + GalNAc); 3171 Da: triantennary monosialo structure (– 2Gal + 2GalNAc); 3212 Da: triantennary monosialo structure (– 3Gal + 3GalNAc); 3451 Da: triantennary structure; 3492 Da: triantennary disialo structure (– Gal + GalNAc); 3533 Da: triantennary disialo structure (– 2Gal + 2GalNAc); 3621 Da: tetraantennary monosialo structure (– 2Gal + 2GalNAc); 3662 Da: tetraantennary monosialo structure (– 3Gal + 3GalNAc); 3813 Da: triantennary trisialo structure; 3941 Da: tetraantennary disialo structure (– Gal + GalNAc); 3983 Da: tetraantennary disialo structure (– 2Gal + 2GalNAc); 4024 Da: tetraantennary disialo structure (– 3Gal + 3GalNAc); 4262 Da: tetraantennary trisialo structure; 4303 Da: tetraantennary trisialo structure (– Gal + GalNAc); 4345 Da: tetraantennary trisialo structure (– 2Gal + 2GalNAc); 4624 Da: tetraantennary tetrasialo structure; 4665 Da: tetraantennary tetrasialo structure (– Gal + GalNAc); 4707 Da: tetraantennary tetrasialo structure (– 2Gal + 2GalNAc).

Table 1. Galactose and GalNAc derivatives in rhuEPO N-glycans detected by methylation analysis

Peracetylated derivative of	BHK-21A wild-type	EPO-ST6NI	Substituted at position
Galacitol			
2,3,4,6-Tetra-*O*-methyl-	+	+	terminal
2,4,6-Tri-*O*-methyl-	++++	+++	3
2,3,4-Tri-*O*-methyl-	n.d.*	++	6
2-*N*-Methylacetamido-2-deoxygalacitol			
3,4,6-Tri-*O*-methyl-	++	+	terminal
4,6-Di-*O*-methyl-	n.d.	n.d.	3
3,4-Di-*O*-methyl-	n.d.	+	6

* n.d. not detected

cans were isolated as described under Material and Methods. Figure 2 shows a representative SDS-PAGE analysis of the purified rhuEPO before and after PNGase F digestion. Coomassie staining of the completely de-N-glycosylated rhuEPO revealed two bands at an apparently molecular mass of 21 and 20 kDa, representing the EPO protein with and without O-glycosylation at Ser126 (Nimtz et al., 1993).

For structural analysis of rhuEPO oligosaccharides complementary methods, such as HPAE-PAD, methylation analysis and MALDI/TOF-MS were used. HPAE-PAD-mapping of the neutral glycans (see Figure 3) revealed a significantly higher sialylation state of N-glycans isolated from rhuEPO produced by cells after transfection with ST6N. The ratios of peak areas corresponding to NeuAc and total oligosaccharides were 1:1.3 for glycans from EPO40 wild-type cells and 1:1 for glycans from EPO-ST6NI as calculated from the corresponding peak areas. Except for the increased NeuAc peak the elution profile of the glycans was almost identical for both rhuEPO N-glycan preparations (see Figure 3).

These results were further confirmed by MALDI/TOF-MS analysis of the permethylated native oligosaccharides (see Figure 4). The data obtained for rhuEPO oligosaccharides produced from transfected EPO-ST6NI cells show an increase of the signals detected in the high mass region when compared to the data obtained for rhuEPO glycans of the parent cell line, indicating a generally higher sialylation of the glycans in rhuEPO from ST6N-transfected cells. rhuEPO N-glycans from EPO40 wild-type cells contain a large amount of unsialylated structures. The molecular ion signal at m/z 2360 corresponding to an unsialylated diantennary structure with two terminal GalNAc residues predominates. rhuEPO oligosaccharides from α2,6-sialyltransferase transfected EPO-ST6NI cells show intense molecular ion signals corresponding to the mono- and disialylated derivatives of this structure (m/z 2721 and 3083, respectively), and a concomitant decrease of the unsialylated derivative at m/z 2360. Sialylated structures of the diantennary glycan with two terminal GalNAc residues were not detected in rhuEPO N-glycans from EPO40 wild-type cells. Similarly, tetraantennary tetrasialylated glycans with one GalNAc residue (m/z 4665), tetraantennary trisialylated glycans with two GalNAc residues (m/z 4345) as well as tetraantennary disialylated glycans with three GalNAc residues (m/z 4024) are detectable in glycans from the product after transfection of cells with ST6N which were not observed in rhuEPO-oligosaccharides from wild-type cells (compare Figure 4 and its legend).

The data obtained after methylation analysis are in agreement with the results obtained by MALDI/TOF-MS and clearly corroborate the expected α2,6-sialylation of rhuEPO N-glycans after transfection with ST6N. Table 1 compares the relative amounts of the detected partially methylated monosaccharide derivatives of Gal and GalNAc. After transfection of EPO40 cells with ST6N, 3-substituted Gal derivatives (2,4,6-Tri-*O*-methyl-galacitol) as well as 6-substituted Gal derivatives (2,3,4-Tri-*O*-methyl-galacitol) were detected indicating NeuAc being linked α2,3 as well as α2,6 to Gal residues. Confirming the substrate specificity of the endogenous α2,3-

sialyltransferase, only terminal GalNAc (3,4,6-Tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalacitol) was found in rhuEPO N-glycans of EPO40 wild-type cells, whereas after transfection with ST6N also the 6-substituted GalNAc derivative (3,4-Tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalacitol) was detected thus verifying unequivocally the α2,6 transfer activity of the newly introduced human enzyme towards GalNAc residues. Therefore, the observed increase in NeuAc content of the rhuEPO N-glycans obtained after transfection of the BHK-21A cells with the human ST6N clearly results from the substrate specificity of the human enzyme acting on both, Gal as well as on GalNAc residues. These *in vivo* data for the human α2,6-sialyltransferase are in agreement with the previously reported *in vitro* substrate specificity studies with the bovine colostrum α2,6-sialyltransferase which recognizes GalNAc(β1–4)GlcNAc acceptors in addition to Gal(β1–4)GlcNAc units (Nemansky and Van den Eijnden, 1992). The *in vitro* activity of the rat liver α2,6-sialyltransferase towards GalNAc(β1–4)GlcNAc-OMe acceptors was found to be 48% of that towards Gal(β1–4)GlcNAc-OMe acceptors (Hokke *et al*., 1993).

In view of the high ST6N mRNA levels which are paralleled by high enzyme activities in EPO-ST6NI cells compared to data of the α2,3-sialyltransferase one would expect significantly higher amounts of α2,6 modified Gal or GalNAc residues. However, data reported from the literature for bovine α2,6-sialyltransferase indicate that this enzyme has low activity with high antennary glycans which predominate in EPO compared to biantennary oligosaccharides (Nemansky et al., 1995). In addition, the human α2,6-sialyltransferase has been reported to have a high preference for the Man(α1-3)Man branch of complex diantennary N-glycans (Grabenhorst et al., 1995).

To our knowledge the results reported in the present study provides for the first time *in vivo* evidence that the human α2,6-sialyltransferase recognizes the terminal GalNAc(β1–4)GlcNAc-R structures as a substrate. Therefore, it is likely that this enzyme is also involved in the synthesis of the NeuAc(α2–6))GalNAc(β1–4)GlcNAc-R sequence found on N-glycans from different human tissues, e.g. human lutropin (Weisshaar et al., 1991) and human urokinase (Bergwerff et al., 1994).

The stable expression of the recombinant genes in the EPO-ST6NI cell line was demonstrated in a culture process using a 2.5L stirred perfusion bioreactor. The analysis of the rhuEPO N-glycans from the product obtained confirmed the stability of the cell line over the culture period of up to 18 days without significant changes in ST6N mRNA levels or sialyltransferase activity assayed *in vitro*.

Conclusion

By transfection of the BHK-21A EPO40 cell line with the human CMP-NeuAc:Gal(β1–4)GlcNAc-R sialyltransferase we have successfully genetically engineered a novel host cell line with *human-type* glycosylation properties which is characterized by the capability to transfer NeuAc in α2,6-linkage to terminal Gal and GalNAc residues of N-glycans from recombinant glycoproteins in addition to the common α2,3 NeuAc transfer to Gal termini by the endogenous α2,3-sialyltransferase of this host as follows:
Terminal motifs of oligosaccharides synthesized by EPO40 wild-type cells:

NeuAc(α2–3)**Gal**(β1–4)GlcNAc-R
GalNAc(β1–4)GlcNAc-R
Gal(β1–4)GlcNAc-R

Terminal motifs of oligosaccharides synthesized by cells transfected with human ST6N:

NeuAc(α2–6)**GalNAc**(β1–4)GlcNAc-R
NeuAc(α2–3/6)**Gal**(β1–4)GlcNAc-R
GalNAc(β1–4)GlcNAc-R
Gal(β1–4)GlcNAc-R

The transfection of hererologous mammalian host cell lines with plasmids encoding glycosyltransferases of defined specificity provides a valuable tool for the manipulation of their glycosylation machinery towards the production of *human-type* glycosylated products. The construction of such novel host cell lines will aid to improve the *in vivo* safety and stability of biotechnologically produced recombinant proteins destined for clinical use. Eventually glycoprotein products obtained from such manipulated cell lines may exhibit novel advantageous pharmacological properties.

Similarly, other human glycosyltransferases e.g. fucosyltransferases that add fucose to outer antennae of oligosaccharides could be introduced into BHK cells resulting in producer cell lines that allow the isolation of glycoprotein products containing Lewis^x or sialyl-Lewis^x motifs which are ligands for the selection family of receptors.

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