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Enhanced sialylation of recombinant human erythropoietin in Chinese hamster ovary cells by combinatorial engineering of selected genes

Young-Dok Son, Yeon Tae Jeong, Seung-Yeol Park, and Jung Hoe Kim[1]

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 335 Gwahangno, Yuseong-gu, Daejeon 305-701, Republic of Korea

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Therapeutic glycoproteins with exposed galactose (Gal) residues are cleared rapidly from the bloodstream by asialoglycoprotein receptors in hepatocytes. Various approaches have been used to increase the content of sialic acid, which occupies terminal sites of *N*- or *O*-linked glycans and thereby increases the half-life of therapeutic glycoproteins. We enhanced sialylation of human erythropoietin (EPO) by genetic engineering of the sialylation pathway in Chinese hamster ovary (CHO) cells. The enzyme GNE (uridine diphosphate-*N*-acetyl glucosamine 2-epimerase)/MNK (*N*-acetyl mannosamine kinase), which plays a key role in the initial two steps of sialic acid biosynthesis, is regulated by cytidine monophosphate (CMP)-sialic acid through a feedback mechanism. Since sialuria patient cells fail in regulating sialic acid biosynthesis by feedback mechanism, various sialuria-like mutated rat GNEs were established and subjected to in vitro activity assay. GNE/MNK-R263L-R266Q mutant showed 93.6% relative activity compared with wild type and did not display feedback inhibition. Genes for sialuria-mutated rat GNE/MNK, Chinese hamster CMP-sialic acid transporter and human α2,3-sialyltransferase (α2,3-ST) were transfected simultaneously into recombinant human (rh) EPO-producing CHO cells. CMP-sialic acid concentration of engineered cells was significantly (>10-fold) increased by sialuria-mutated GNE/MNK (R263L-R266Q) expression. The sialic acid content of rhEPO produced from engineered cells was 43% higher than that of control cells. Ratio of tetra-sialylated glycan of rhEPO produced from engineered cells was increased ∼32%, but ratios of asialo- and mono-sialylated glycans were decreased ∼50%, compared with control. These findings indicate that sialuria-mutated rat GNE/MNK effectively increases the intracellular CMP-sialic acid level. The newly constructed host CHO cell lines produced more highly sialylated therapeutic glycoproteins through overexpression of sialuria-mutated GNE/MNK, CMP-SAT and α2,3-ST.

Keywords: CMP-sialic acid transporter / erythropoietin / GNE/MNK / sialylation / sialyltransferase

Introduction

Glycosylation is a frequent and important type of post-translational modification in eukaryotic cells. A variety of complex glycan structures affect many properties of glycoproteins, including stability, immunogenicity, enzymatic activity, protease resistance, secretion and in vivo half-life (Goochee et al. 1991; Varki 1993; Jenkins and Curling 1994; Walsh and Jefferis 2006). Most proteins used for therapy of human diseases are glycosylated, and the glycan structures have been shown to affect therapeutic quality (Apweiler et al. 1999). Many therapeutic proteins are produced in mammalian cells such as CHO (Chinese hamster ovary), whose glycosylation pathways are similar to those of human cells.

Asparagine-linked glycan structures, termed *N*-linked glycans, display complex-type structures in mammalian cells (Jenkins et al. 1996). Many studies have shown that qualities of therapeutic glycoproteins are affected by variable branching structures, *N*-acetyl glucosamine (GlcNAc) bisecting, fucosylation and terminal sialic acid capping (Egrie and Browne 2001; Arnold et al. 2007). In vivo half-life of therapeutic glycoproteins, in particular, is determined mainly by sialic acid capping in the end sites of *N*-linked glycans (Ngantung et al. 2006). Many non-sialylated glycoproteins have significantly shorter in vivo half-lives, because exposed Gal residues are recognized and captured by asialoglycoprotein receptors in hepatocytes (Fukuda et al. 1989; Weiss and Ashwell 1989). By maximizing the sialic acid content of therapeutic glycoproteins, we may increase their in vivo half-life.

Biosynthesis of sialic acid in mammalian cells takes place in the cytosol, catalyzed by several enzymes (Figure 1). Uridine diphosphate (UDP)-GlcNAc is initially epimerized to *N*-acetyl mannosamine (ManNAc) by UDP-GlcNAc 2-epimerase (GNE), and ManNAc is phosphorylated to ManNAc-6-phosphate by ManNAc kinase (MNK). These two

[1]To whom correspondence should be addressed: Tel: +82-42-350-2614; Fax: +82-42-350-5614; e-mail: kimjh@kaist.ac.kr

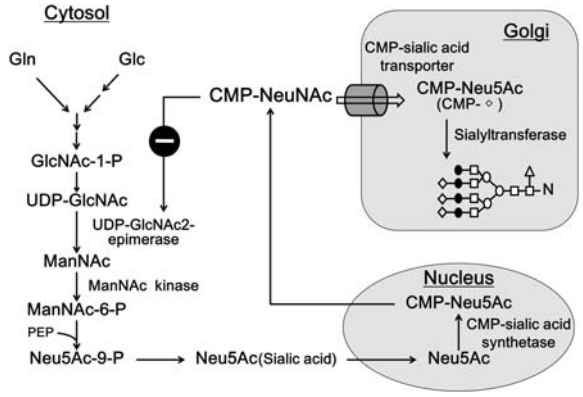

Fig. 1. CMP-sialic acid (NeuNAc) biosynthesis pathway and sialylation of *N*-linked glycans, in mammalian cells. CMP-sialic acid, the active substrate of sialyltransferase, inhibits activity of GNE, which acts on the initial step of the CMP-sialic acid biosynthesis pathway through feedback regulation.

enzymes are integrated into a single bifunctional enzyme, GNE/MNK (EK), in mammalian cells (Stäsche et al. 1997; Reinke et al. 2009). ManNAc-6-phosphate is condensed with phosphoenolpyruvate to synthesize *N*-acetyl neuraminic acid (NeuNAc)-9-phosphate, catalyzed by sialic acid-9-phosphate synthase (Roseman et al. 1961; Warren and Felsenfeld 1961; Viswanathan et al. 2005), and NeuNAc (sialic acid) is synthesized by dephosphorylation. Sialic acid is activated to cytidine monophosphate (CMP)-sialic acid, a sugar nucleotide donor, in the nucleus by CMP-sialic acid synthetase (Kean 1970). CMP-sialic acid is transported to Golgi by CMP-sialic acid transporters (SATs; Wong et al. 2006) and is used there for sialylation of glycans by sialyltransferases (Colley et al. 1989).

In this biosynthetic pathway, GNE is regulated by feedback inhibition of intracellular CMP-sialic acid (Kornfeld et al. 1964). Many groups have attempted to increase the sialylation of therapeutic glycoproteins by increasing the intracellular CMP-sialic acid pool, through supplementation of ManNAc in culture medium or overexpression of CMP-sialic acid synthetase. The intracellular CMP-sialic acid level was greatly increased by supplementation of ManNAc; however, this reagent is too expensive to be utilized for industrial production of therapeutic glycoproteins (Gu and Wang 1998; Baker et al. 2001). CMP-sialic acid synthetase overexpression caused only limited increase in intracellular CMP-sialic acid, because of the feedback regulation of GNE by CMP-sialic acid (Jeong et al. 2009).

In patients with sialuria, a rare congenital disorder of sialic acid metabolism, feedback inhibition of GNE by CMP-sialic acid is absent, leading to excessive synthesis of free sialic acid, which is accumulated in cytoplasm and secreted into urine (Ferreira et al. 1999; Enns et al. 2001). Genetic analysis of sialuria patients identified three point mutations (R263L, R266Q and R266W) within the allosteric site of *GNE* (Ferreira et al. 1999; Seppala et al. 1999; Leroy et al. 2001). Sialic acid concentration in insect cells and CHO cells was increased by overexpression of sialuria-mutated (R263L-mutated) rat GNE/MNK (Viswanathan et al. 2005; Bork et al. 2007).

In the present study, we analyzed the activities of various sialuria-mutated rat GNEs and selected those which maintained high activity even when CMP-sialic acid concentration was high. Human erythropoietin (hEPO) was used as a model protein. This hematopoietic stimulating hormone, produced in the adult kidney, has been used for treatment of various diseases, including renal failure, AIDS and cancer (Eschbach et al. 1987; Markham and Bryson 1995; Cazzola et al. 1997; Sowade et al. 1998). hEPO has three N-linked glycosylation sites (Asn 24, Asn 38 and Asn 83) and one O-linked glycosylation site (Ser 126; Takeuchi et al. 1988). The importance of sialylation in *N*-linked glycans is well documented, and various approaches have been employed to maximize sialylation and to minimize the heterogeneity of *N*-linked glycan structures (Weikert et al. 1999; Egrie and Browne 2001; Bork et al. 2007).

Our previous study demonstrated increased sialylation of recombinant hEPO (rhEPO) produced by CHO cells following transfection with three genes encoding human CMP-sialic acid synthetase, Chinese hamster CMP-SAT and human α2,3-sialyltransferase (α2,3-ST; Jeong et al. 2009). In the present study, CHO cells were transfected with sialuria-mutated rat GNE/MNK selected from in vitro activity assay, instead of CMP-sialic acid synthetase, leading to intracellular CMP-sialic acid levels as high as those caused by supplementation of ManNAc. Chinese hamster CMP-SAT and human α2,3-ST were overexpressed simultaneously, strengthening the sialylation pathway of rhEPO-producing CHO cells.

Results

Comparative activities of various rat GNE mutants

Rat *Gne* was cloned from liver tissue by reverse transcription (RT)–polymerase chain reaction (PCR) as described in *Materials and methods*. Rat *Gne* sequence showed ~88% homology with human *GNE* sequence (GenBank® accession number NM001128227), and putative allosteric sites were conserved. We introduced sialuria-like point mutations into cloned rat *Gne* and determined activities of the resulting mutants.

Three point mutations (R263L, R266Q and R266W) were described previously from genetic analysis of sialuria patients (Ferreira et al. 1999; Seppala et al. 1999; Leroy et al. 2001). We introduced these three single-point mutations, and two combinatorial double-point mutations (R263L-R266Q and R263L-R266W), into cloned rat *Gne* by site-directed mutagenesis. His-tagged wild type and five kinds of sialuria-mutated rat GNE/MNK were overexpressed in *E. coli* BL21 cells and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose, as described in *Materials and methods*. Activities of various sialuria-mutated GNEs were measured in the presence of CMP-sialic acid (Figure 2A). Activity of wild-type rat GNE decreased significantly when concentration of CMP-sialic acid increased, as expected. All the sialuria-mutated rat GNEs maintained their activity in the presence of 150 μM CMP-sialic acid, but their specific activities differed significantly (Figure 2B). R263L and R266Q mutants showed 62.5% relative activity compared with wild type, whereas R266W mutant had 27.4% activity and R263L-R266W

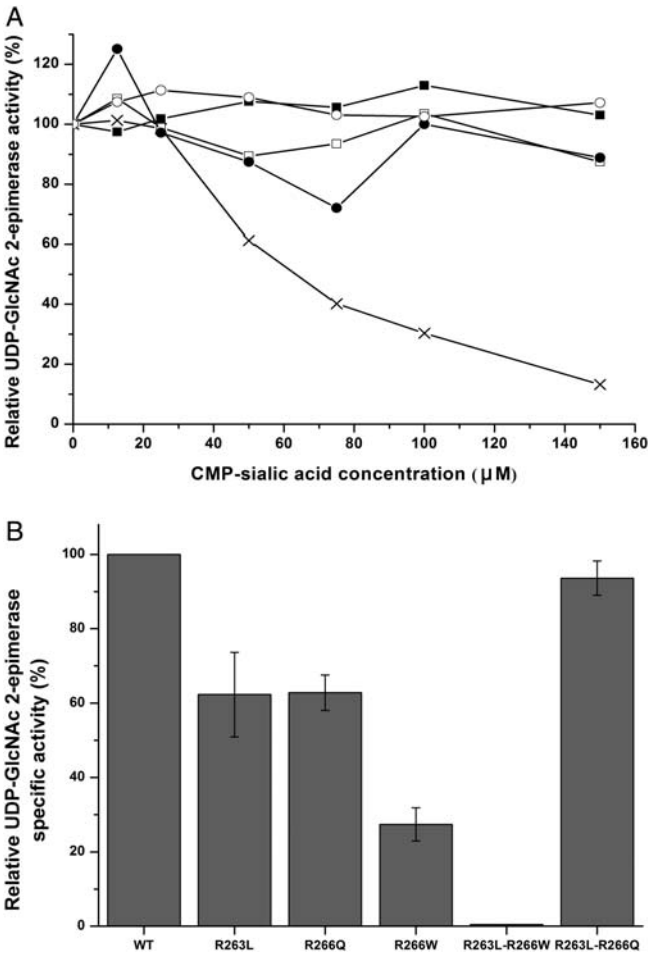

Fig. 2. In vitro analysis of GNE activity. (**A**) Effect of CMP-sialic acid on the activity of wild-type and sialuria-mutated rat GNE. (**B**) Relative specific activity of sialuria-mutated rat GNE. Wild-type and sialuria-mutated rat GNE/MNK were expressed in *E. coli* BL21 cells, purified by Ni-NTA affinity chromatography and assayed for GNE activity as described in *Materials and methods*. Data shown are mean ± SD from duplicate experiments. Crosses, wild type; black squares, R263L mutant; white squares, R266Q mutant; black circles, R266W mutant; white circle, R263L-R266Q mutant.

mutant had only 0.5% activity. R263L-R266Q mutant showed 93.6% relative activity, the highest activity observed among the sialuria-mutated rat GNEs.

Expression of human α2,3-ST, Chinese hamster CMP-SAT and sialuria-mutated rat GNE/MNK in rhEPO-producing CHO cells

Stable cell lines overexpressing sialuria-mutated rat GNE/MNK, Chinese hamster CMP-SAT and human α2,3-ST, simultaneously, were selected using zeocin and hygromycin B. Rat GNE/MNK-R263L-R266Q mutant, selected from in vitro activity assay, was expressed in EC2-1H9-CTSTrEKm cell line. For comparative purposes, rat GNE/MNK-R263L mutant was expressed in EC2-1H9-CTSTmEK cell line. Total RNA of each transfectant was isolated, and transcripts of human α2,3-ST (Figure 3A) and sialuria-mutated rat GNE/MNK (Figure 3B) were detected by RT–PCR. pcSTz and pREP4-rEKh-R263L vectors were used as positive controls

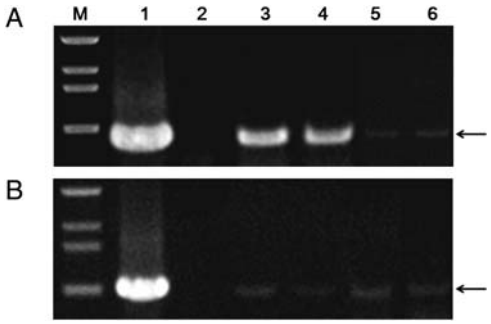

Fig. 3. Total RNA of transfected cells was isolated using TRIzol reagent, and the expression of human α2,3-ST (**A**) and sialuria-mutated rat GNE/MNK (**B**) transcripts was examined by RT–PCR. pcSTz and pREP-rEKh vectors were used as positive controls; total RNA from EC2-1H9 cells was used as a negative control. M, 1 kb marker; lane 1, positive control; lane 2, negative control; lane 3, EC2-1H9-CTSTmEK II-3; lane 4, EC2-1H9-CTSTmEK II-7; lane 5, EC2-1H9-CTSTrEKm 2; lane 6, EC2-1H9-CTSTrEKm 9.

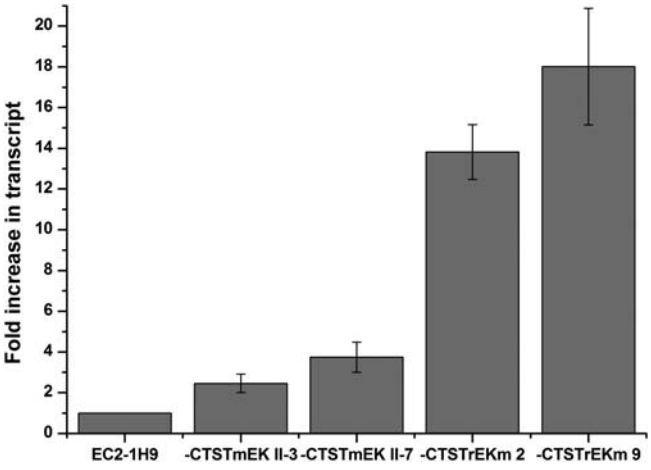

Fig. 4. Detection of total CMP-SAT transcript in selected clones by real-time PCR. Fold increase in CMP-SAT transcript was normalized relative to control cells (EC2-1H9). Data shown are mean ± SD from triplicate experiments.

(Figure 3, lane 1). Human α2,3-ST and sialuria-mutated rat GNE/MNK transcripts were not detected in EC2-1H9 control cells (lane 2). Two clones from each cell line showed expression of both genes (EC2-1H9-CTSTmEK II-3, II-7, and EC2-1H9-CTSTrEKm 2, 9).

Transcript levels of Chinese hamster CMP-SAT were determined quantitatively using real-time PCR. The levels were calculated based on C_t values and normalized relative to the transcript levels of endogenous β-actin genes. Degree of increase in CMP-SAT transcripts relative to control cells ranged from 2.5- to 18.0-fold (Figure 4). Based on the RT–PCR and real-time PCR analyses, we selected four CHO EC2-1H9 cell lines that overexpressed the three genes. In order to assess the improvement of rhEPO sialylation, wild-type EC2-1H9 and the four constructed cell lines were cultured to produce rhEPO.

Quantification of intracellular CMP-sialic acid concentration

Intracellular CMP-sialic acid concentration was quantified to confirm the effect of sialuria-mutated rat GNE/MNK

expression. Cells at exponential growth phase in T-175 cm^2 culture flasks were harvested, and 1.0×10^7 cells were used for the analysis. Extracted sugar nucleotides were separated on CarboPac PA1 column, and sugar nucleotide peaks were detected using a UV detector at Abs_{260}.

Intracellular CMP-sialic acid concentration increased significantly in all transfected cell lines (Figure 5). Cell lines expressing rat GNE/MNK-R263L mutant (-CTSTmEK II-3 and -CTSTmEK II-7) showed up to 5.2- and 4.7-fold increases, compared with control EC2-1H9. In mutant cell lines expressing GNE/MNK-R263L-R266Q (-CTSTrEKm 2 and -CTSTrEKm 9), the concentration was increased 10.5- and 10.7-fold. These results indicate that the feedback inhibition of CMP-sialic acid is overcome by expression of sialuria-mutated rat GNE/MNK. Similar to findings from in vitro activity assay, the cell lines that expressed GNE/MNK-R263L-R266Q (-CTSTrEKm 2 and -CTSTrEKm 9) showed much greater increase in intracellular CMP-sialic acid concentration than cell lines expressing rat GNE/MNK-R263L (-CTSTmEK II-3 and -CTSTmEK II-7).

Production and purification of rhEPO from genetically engineered CHO cells

EC2-1H9 cells, and constructed 4CHO cell lines, were seeded in T-175 cm^2 culture flasks (5.0×10^6 cells/flask). Culture medium was replaced with serum-free medium after 3 days, and cells were cultured for two additional days. The harvested culture medium was concentrated, and buffer changed to PBS by ultrafiltration. rhEPO was purified from concentrated culture medium by immunoaffinity chromatography, as described in *Materials and methods*. The eluate was concentrated and dialyzed by ultrafiltration, and purified rhEPO was analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie Blue staining. No significant differences were observed among the purified rhEPO samples (data not shown).

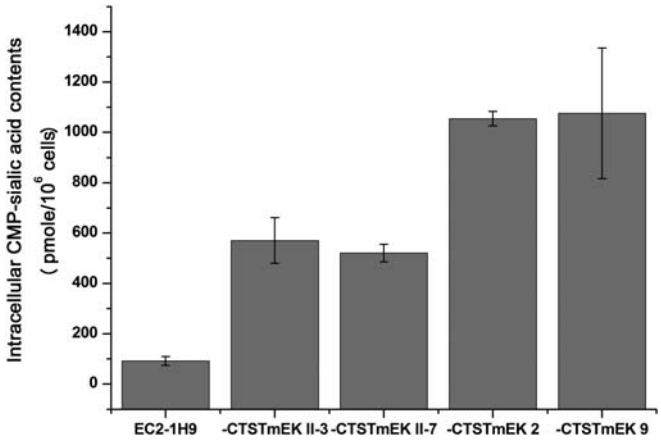

Fig. 5. Intracellular CMP-sialic acid concentration of EC2-1H9 cells and genetically engineered CHO cells. Extracted CMP-sialic acid was separated on Carbopac PA1 column, and its level was normalized relative to the cell number. Data shown are mean ± SD from four independent experiments.

Isoform profiles of rhEPO by isoelectric focusing

A defined amount (10 μg) of purified rhEPO was placed on pH 3–7 range isoelectric focusing (IEF) gel, and rhEPO isoforms were separated based on net electrical charge (Figure 6A). Dramatic shifts of isoform patterns toward the anode end were observed in the IEF gel image. For quantitative comparison of changes in isoform patterns, ratios of band intensity in a specified area were measured using Scion Image program. Intensity ratios of isoforms with pI < 4.5 were compared to determine the increase in rhEPO sialylation (Figure 6B). Ratios increased from 46.0% in EC2-1H9 control rhEPO to 58.4, 57.4 and 51.2% in -CTSTmEK II-3, -CTSTmEK II-7 and -CTSTrEKm 2, respectively. rhEPO from EC2-1H9-CTSTrEKm 9 cells showed a 72.7% increase, which was ~27% higher than that of control rhEPO from EC2-1H9 cells.

Determination of sialic acid content of rhEPO

The sialic acid content of purified rhEPO was analyzed using the *o*-phenylenediamine (OPD)-labeling method (Anumula 1995). Sialic acid was detached from purified rhEPO by mild hydrolysis and then derivatized with OPD. The OPD-labeled sialic acids were separated on reversed-phase C_{18} column by high-performance liquid chromatography (HPLC) and detected by fluorescence detector. hEPO has one O-linked glycosylation site (two sialic acid residues) and three N-linked

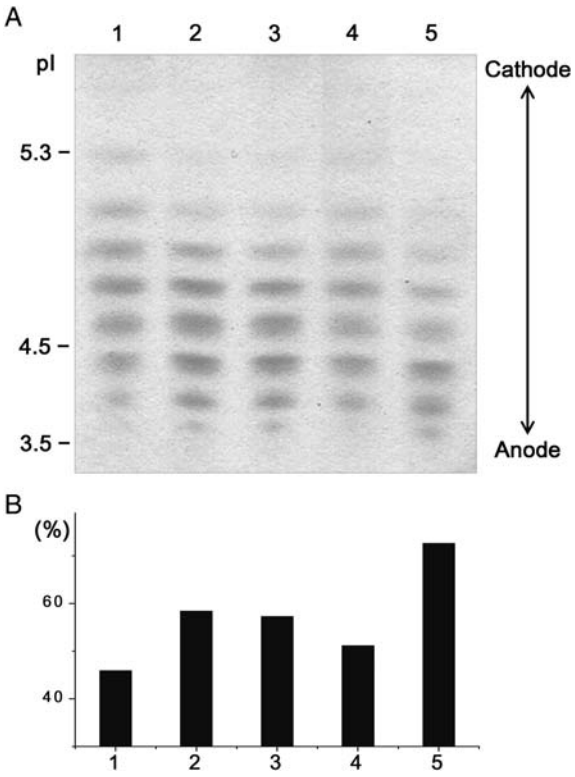

Fig. 6. Comparison of rhEPO isoform patterns by IEF analysis (**A**) and comparison of intensity ratio at the anode end (**B**). Purified rhEPO isoforms were separated on IEF gel (pI 3–7) and visualized by Coomassie Blue staining. Changes in the isoform patterns were compared quantitatively to intensity the ratio of isoforms with pI < 4.5. Lane 1, EC2-1H9; lane 2, EC2-1H9-CTSTmEK II-3; lane 3, EC2-1H9-CTSTmEK II-7; lane 4, EC2-1H9-CTSTrEKm 2; lane 5, EC2-1H9-CTSTrEKm 9.

glycosylation sites (four sialic acid residues). Up to 14 moles of sialic acid can therefore be attached to 1 mol hEPO. We determined that the sialic acid content of rhEPO from EC2-1H9-CTSTmEK II-3 and II-7 was increased ~15 and 20%, respectively, compared with that of EC2-1H9 control (Figure 7). The sialic acid content of rhEPO from EC2-1H9-CTSTrEKm 2 and 9 cells was determined as 8.8 and 9.6 mol sialic acids/mol rhEPO, as increase of ~30 and 43% compared with EC2-1H9 control.

Sialylation profile of N*-linked glycans from rhEPO*

For more precise analysis of changes in sialylation patterns, *N*-linked glycans of purified rhEPO were released by *N*-glycosidase F digestion and then purified and derivatized with 2-aminobenzamide (2-AB). Purified 2-AB-labeled *N*-linked glycans were separated on an anion-exchange column (DEAE-5PW) based on the number of negatively charged sialic acid residues (Figure 8). Peaks of 2-AB-labeled *N*-linked glycans were detected by a fluorescence detector, and sialic acid number was determined by comparison with standard 2-AB-labeled bovine fetuin *N*-glycan (Figure 8, "Bovine Fetuin"). Relative amount for each sialylation profile was determined by the ratio of peak area (Table I). Consistent with results from IEF and sialic acid content analysis, rhEPO from the four engineered cell lines showed significantly increased sialylation. Ratios of asialo- and mono-sialylated *N*-glycans for EC2-1H9-CTSTmEK II-3 and II-7 were decreased ~50% compared with control EC2-1H9, whereas ratios of tri- and tetra-sialylated *N*-glycans were increased ~5 and 12%. For EC2-1H9-CTSTrEKm 9 cells, ratios of asialo- and mono-sialylated *N*-glycans decreased to the same extent as in EC2-1H9-CTSTmEK, but the increase in the ratio of tetra-sialylated *N*-glycans was much greater (~ 32%).

Discussion

Terminal sialic acid residues in *N*-linked glycans of most therapeutic glycoproteins play key roles in the in vivo clearance rate, stability, immunogenicity and enzymatic activity. Many studies have demonstrated the importance of fully sialylated *N*-linked glycans and of consistent production of homogeneous *N*-linked glycan structures, in therapeutic glycoproteins (Egrie and Browne 2001; Ngantung et al. 2006). In the present study, we attempted to enhance sialylation of hEPO from CHO cells by increasing intracellular CMP-sialic acid concentration and by enhancement of sialylation through combinatorial genetic engineering.

Cell lines transfected simultaneously with three genes (encoding human α2,3-ST, Chinese hamster CMP-SAT and human CMP-sialic acid synthetase) were constructed in our previous study (Jeong et al. 2009). Cells in which human CMP-sialic acid synthetase was overexpressed showed a marginal increase in the CMP-sialic acid level, ~150% relative to control cells. Patients with sialuria show high free sialic acid concentration in the cell cytoplasm and urine (Ferreira et al. 1999; Enns et al. 2001). Genetic analysis showed that this phenotypic change is correlated with three point mutations (R263L, R266Q and R266W) on GNE/MNK, which affect the two initial steps of sialic acid biosynthesis (Ferreira et al. 1999; Seppala et al. 1999; Leroy et al. 2001). To further increase the intracellular CMP-sialic acid level, we eliminated feedback inhibition of GNE by CMP-sialic acid, by introducing sialuria-mutated GNE/MNK. In vitro activity of sialuria-mutated rat GNE was compared with that of enzyme which was point mutated with combinatorial changes. R263L-R266Q double point-mutated enzyme showed the highest epimerase activity, similar to that of wild-type GNE/MNK (Figure 2B). Activity of this mutant was not inhibited by increase in the CMP-sialic

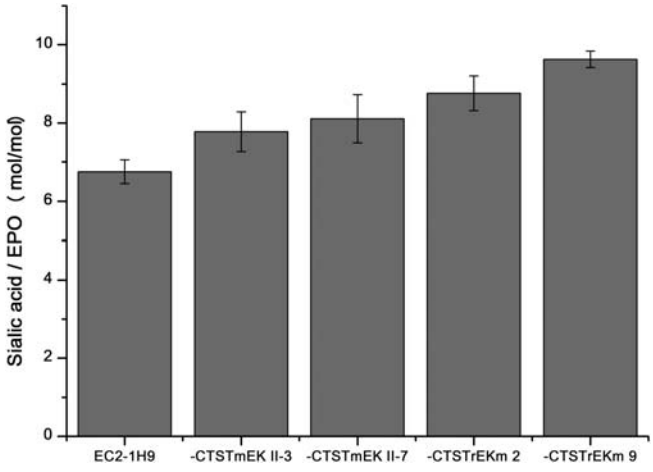

Fig. 7. Sialic acid content of rhEPO produced by EC2-1H9 cells and genetically engineered CHO cells, determined by the OPD-derivatization method. Data shown are mean ± SD from three independent experiments.

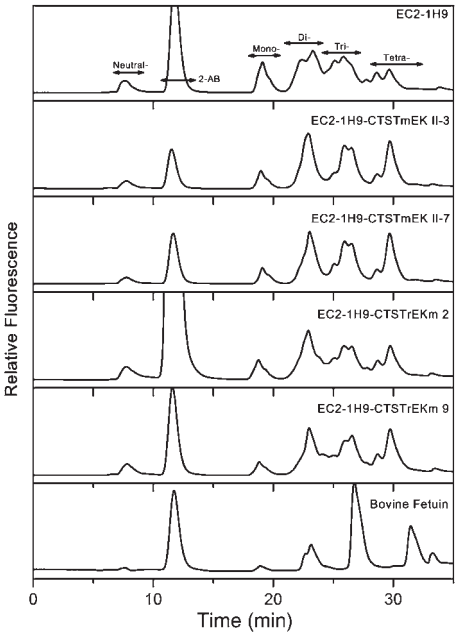

Fig. 8. Anion-exchange HPLC analysis of 2-AB-labeled *N*-linked glycans from rhEPO. *N*-Linked glycans from purified rhEPO were derivatized with 2-AB and then separated on anion-exchange column (DEAE-5PW; Tosoh) according to the number of sialic acid residues. Peaks of sialylation profiles were identified by comparison to standard 2-AB-bovine fetuin *N*-linked glycan library (Glyko®). 2-AB peak indicates the non-bounded 2-AB reagent.

Table I. Relative amounts of sialylated *N*-glycans in rhEPO, analyzed by anion-exchange HPLC (DEAE-5PW; Tosoh)

Sialylated glycans	Relative amounts of sialylated glycans (%)				
	EC2-1H9	-CTSTmEK II-3	-CTSTmEK II-7	-CTSTrEKm 2	-CTSTrEKm 9
Neutral (asialo-)	12.25	4.63	5.83	7.63	6.71
Mono-	20.50	9.02	7.98	13.74	9.01
Di-	30.03	33.03	29.70	32.82	27.73
Tri-	23.27	27.55	28.33	23.87	24.53
Tetra-	13.97	25.79	28.17	21.95	32.03

Data shown are mean values from duplicate experiments.

acid level (Figure 2A). Activity of rat GNE/MNK-R263L mutant was only 62.5% that of wild type (Figure 2B), although this mutant enzyme was used previously to increase the CMP-sialic acid level in insect cells and CHO cells (Viswanathan et al. 2005; Bork et al. 2007). These findings suggest that rat GNE/MNK-R263L-R266Q mutant could be used effectively to increase the intracellular CMP-sialic acid level, through genetic engineering.

Next, rat GNE/MNK-R263L-R266Q and GNE/MNK-R263L mutants were expressed in rhEPO-producing CHO cells, in order to increase the CMP-sialic acid level. As expected from in vitro activity assay results, cell lines expressing GNE/MNK-R263L-R266Q mutant, when compared with GNE/MNK-R263L mutant, showed a significantly higher CMP-sialic acid level (Figure 5). These findings are consistent with those of previous studies in which the CMP-sialic acid level in CHO cells was significantly increased by addition of ManNAc to the culture medium (Gu and Wang 1998; Baker et al. 2001). In those studies, however, the increased CMP-sialic acid level did not cause significant increase in sialylation of target glycoproteins (recombinant interferon-γ and TIMP-1). In CHO cells, overexpression of sialuria-mutated rat GNE/MNK by itself did not significantly increase the sialylation of rhEPO (unpublished data). Increased CMP-sialic acid in cytosol is presumably transported to Golgi lumen for effective use in sialylation of glycoproteins. We therefore strengthened the sialylation pathway by overexpression of human α2,3-ST and Chinese hamster CMP-SAT, simultaneously with the expression of sialuria-mutated rat GNE/MNK. Transport of CMP-sialic acid from cytosol to Golgi lumen was facilitated by overexpression of CMP-SAT, and sialylation of *N*-linked glycans was improved by overexpression of α2,3-ST.

We used various approaches to analyze sialylation of rhEPO produced by these engineered CHO cell lines. Isoforms of rhEPO were initially subjected to IEF analysis (Figure 6). rhEPO isoforms from the engineered cell lines showed a distinct shift to anode ends, due to the enhanced sialylation of rhEPO. A number of rhEPO isoforms were observed in the IEF gel, suggesting that rhEPO still has heterogeneous sialylated glycan structures. Next, sialylation profiles of *N*-linked glycans from rhEPO were analyzed using the 2-AB-labeling method. Neutral and mono-sialylated *N*-linked glycans were decreased ~50% when compared with those from control EC2-1H9, and tetra-sialylated *N*-linked glycans were increased strongly in rhEPO from all engineered cell lines (Table I). Di- and tri-sialylated *N*-linked glycans did not show a significant change in any of the cell lines. These findings, taken together, indicate that the factors that limit complete sialylation of glycoproteins are retained in the engineered cell lines.

Since the terminal Gal residues of *N*-linked glycans are used as substrates for α2,3- and α2,6-ST, complete galactosylation of *N*-linked glycans is a prerequisite for complete sialylation (Hossler et al. 2009). Incomplete galactosylation of *N*-linked glycans was reported as a limiting factor for complete sialylation (Weikert et al. 1999; Warner 2001). Galactosylation of *N*-linked glycans is catalyzed by β1,4-galactosyltransferase (GalT), using UDP-Gal as a substrate. Exogenous supplementation of Gal and overexpression of β1,4-GalT, significantly decreased the terminal branches with GlcNAc of glycoproteins such as GP1-IgG (Glycoprotein 1-IgG fusion protein) and TNFR-IgG (tissue necrosis factor receptor-IgG1 fusion protein) (Weikert et al. 1999; Warner 2001). Co-expression of α2,3-ST and β1,4-GalT in CHO cells showed much higher increase in sialylation of rhEPO than α2,3-ST only expressed CHO cells in our previous study (Jeong et al. 2008).

Limitation of sialyltransferase activity is another possible cause of incomplete sialylation in *N*-linked glycans of glycoproteins. In the case of highly branched or highly sialylated glycan structures, sialyltransferase activity may be inhibited, by steric hindrance from complex *N*-linked glycan branching structures, and the repulsive force of negatively charged sialic acid (Nemansky et al. 1995). Sialyltransferase activity was significantly reduced when mono-sialylated rather than asialo-*N*-acetyllactosamine was used as a substrate (Joziasse et al. 1987). In the present study, levels of asialo- and mono-sialylated *N*-glycans were reduced, whereas levels of di- and tri-sialylated *N*-glycans were not significantly changed, in the engineered cell lines. We predict that sialyltransferases having consistently high activity, and no specificity for highly branched or highly sialylated *N*-linked glycans, will be found essential to achieve complete sialylation.

The new double point-mutated (R263L-R266Q) rat GNE/MNK displayed high epimerase activity in comparison with wild type, without feedback inhibition by CMP-sialic acid. The intracellular CMP-sialic acid level in CHO cells was significantly increased by overexpression of rat GNE/MNK-R263L-R266Q mutant to the same level as by the ManNAc supplementation approach. Sialylation of rhEPO was greatly increased by simultaneous overexpression of rat GNE/MNL-R263L-R266Q, human α2,3-ST and Chinese hamster CMP-SAT. The rat GNE/MNK-R263L-R266Q mutant should be useful to enhance the sialylation pathway in

insect cells or yeast cells designed to produce humanized therapeutic glycoproteins.

Materials and methods

Cloning of rat GNE/MNK and introduction of sialuria-like mutations

Rat GNE/MNK was cloned from rat liver tissue, based on the sequence reported previously (GenBank® accession number BC062011). Total RNA of rat liver tissue was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA), and *Gne* cDNA strands were synthesized by RT–PCR (AccuPower RT-PCR PreMix; Bioneer, Daejeon, Korea). For rat *Gne* cloning, forward (5′-ATG GAG AAG AAC GGG AAT AAC CGG AAG C-3′) and reverse (5′-CTA GTG GAT CCT GCG GGT CGT GTA GTC C-3′) primers were used. Cloned wild-type rat *Gne* was inserted into pGEM-T Easy Vector (Promega, Madison, WI), and sialuria-like point mutations were introduced using QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Five types of sialuria-mutated rat *Gne* were obtained using the following primers: R263L mutant, 5′-GCA AGG AGA TGG TTC TAG TGA TGC GGA AGA AGG-3′; R266Q mutant, 5′-GCA AGG AGA TGG TTC GAG TGA TGC AGA AGA AGG-3′; R266W mutant, 5′-GCA AGG AGA TGG TTC GAG TGA TGT GGA AGA AGG-3′; R263L-R266Q mutant, 5′-GCA AGG AGA TGG TTC TAG TGA TGC AGA AGA AGG-3′; R263L-R266W mutant, 5′-GCA AGG AGA TGG TTC TAG TGA TGT GGA AGA AGG-3′. Sequences of mutated genes were verified by di-deoxy sequencing. To attach C-terminal His-tag for overexpression in *Escherichia coli*, sialuria-mutated rat *Gne* was inserted into pET-21a(+) vector (Invitrogen).

Overexpression and purification of sialuria-mutated rat GNE/MNK in E. coli

The constructed vectors were expressed in *E. coli* BL21 (RBC, Taipei, Taiwan). Wild-type and sialuria-mutated rat GNE/MNK were produced and purified as described previously (Blume et al. 2004). In brief, transformed cells were cultured in 50 mL Luria-Bertani (LB) medium at 37°C, with rotary shaking at 250 rpm. When OD_{600} of cultured cells reached 0.6–0.8, 0.03 mM isopropyl-β-D-thiogalactopyranoside was inoculated to overexpress the enzymes. Cells were then grown at 18°C, 200 rpm for 8 h, centrifuged at 4000 × *g* for 20 min at 4°C, resuspended in 2.5 mL PBS with 1 mM DTT (dithiothreitol), 1 mM phenylmethylsulfonyl fluoride, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM UDP, lysed with a sonic cell disruptor (Vibra Cell 130; Sonics & Materials, Newtown, CT) and centrifuged at 20,000 × *g* for 30 min at 4°C. The lysate supernatant was mixed with Ni-NTA agarose (Qiagen, Hilden, Germany) at room temperature for 1 h. The resins were packed in mini-columns, bound proteins were washed with 10 mL of 50 mM Na_2HPO_4, pH 8.0, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM UDP, 300 mM NaCl and 20 mM imidazole, and His-tagged rat GNE/MNK was eluted with 3 mL of the same solution except with 100 mM imidazole.

Determination of activity of sialuria-mutated rat GNE

Activity of purified rat GNE was determined as described previously (Blume et al. 2002). In brief, 100 µL purified rat GNE/MNK was mixed with 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$ and 1 mM UDP-GlcNAc in a 200 µL reaction volume. Various concentrations of CMP-sialic acid (Sigma , St. Louis, USA) were added in reaction mixtures to test inhibitory effect on GNE activity. The mixtures were reacted for 30 min at 37°C, boiled for 1 min to stop the reaction and centrifuged for 1 min at 20,000 × *g*. Produced ManNAc was analyzed by the Morgan–Elson method (Reissig et al. 1955). In brief, 150 µL of sample was mixed with 30 µL of 0.8 M borate, pH 9.1, boiled for 3 min, mixed with 800 µL DMAB solution (1% (w/v) 4-dimethylamino benzaldehyde in acetic acid, 1.25% 10 M HCl) and incubated for 30 min at 37°C. Absorbance of sample was detected at 578 nm. One enzyme unit was defined as the formation of 1 µmol ManNAc per minute at 37°C. Specific activity was expressed as milliunit per milligram protein. Protein concentration of eluate was determined using Quant-iT™ protein assay kit (Invitrogen).

Transfection and construction of stable cell lines

Rat GNE/MNK-R263L-R266Q mutant and rat GNE/MNK-R263L mutant were inserted into pREP4 mammalian cell expression vector (Invitrogen), for construction of pREP4-rEKh-R263L-R266Q and pREP4-rEKh-R263L vector, respectively. Our previous study (Jeong et al. 2009) included construction of two vectors, pcSTz and pcCSATz, which express human α2,3-ST and Chinese hamster CMP-SAT, respectively.

EC2-1H9, an rhEPO-producing CHO cell line, was provided by Dr Hyo Jeong Hong (Antibody Engineering Research Unit, Korea Research Institute of Bioscience and Biotechnology, Yuseong-gu, Daejeon, Korea). pcSTz, pcCSATz and pREP4-rEKh-R263L (or pREP4-rEKh-R263L-R266Q) vectors were introduced simultaneously into EC2-1H9 cells using Lipofectamine™ LTX and PLUS™ reagents (Invitrogen), according to the manufacturer's instructions.

Transfected cells were seeded (10 cells $well^{-1}$) in 96-well tissue culture plates (Nunc, Roskilde, Denmark), and stable cell lines were selected for 2 weeks in modified Eagles medium-α (MEM-α; Gibco, Grand Island, NY) supplemented with 10% dFBS (dialyzed fetal bovine serum; SAFC), 3.5 g L^{-1} glucose, 500 µg mL^{-1} zeocin (Invitrogen), 400 µg mL^{-1} hygromycin B (Invitrogen), 20 nM MTX (methotrexate; Sigma) and 1% Ab-Am (antibiotic-antimycotic solution; Gibco) in a humidified atmosphere containing 5% CO_2 at 37°C. Two cell lines were constructed, expressing pcSTz, pcCSATz and pREP4-rEKh-R263L (or pREP4-rEKh-R263L-R266Q) and were, respectively, termed EC2-1H9-CTSTmEK and EC2-1H9-CTSTrEKm.

Analysis of human α2,3-ST transcript and sialuria-mutated rat GNE/MNK transcript expression by RT–PCR

Total RNA of recombinant CHO cells was extracted using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. RT of 1.0 µg RNA to cDNA was performed

using AccuPower RT-PCR PreMix (Bioneer). The cDNA was used as template to confirm mRNA transcripts of human α2,3-ST and sialuria-mutated rat GNE/MNK by PCR. To amplify human α2,3-ST, 5′-GAG GAG GAC TCC AAT TCA GTG GTT C-3′ (forward) and 5′-CAT AGC CAA ATC CTG CGA CTG CCA C-3′ (reverse) primers were used. To amplify sialuria-mutated rat GNE/MNK, 5′-GGT GAC CAC CGA CAT TAA GCA TTC C-3′ (forward) and 5′-GAG CGT CAC AAA GTT CTC CTG TCC-3′ (reverse) primers were used. Amplified genes were separated on 0.8% agarose gel and visualized by ethidium bromide staining.

Analysis of the Chinese hamster CMP-SAT transcript level by real-time PCR

Total cDNA of each cell line prepared using RT-PCR, was used as template for real-time PCR and performed using the MyiQ™ real-time detection system (Bio-Rad, Hercules, CA). PCR conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. The reaction was performed in a 20 μL mixture containing 2× Fast Start SYBR Green Mater (Roche, Mannheim, Germany), 10 pmol forward and reverse primers and 5 μL of 1:10 diluted cDNA template. To analyze the transcript level of Chinese hamster CMP-SAT, 5′-TGA TAA GTG TTG GAC TTT TAG C-3′ (forward) and 5′-CTT CAG TTG ATA GGT AAC CTG G-3′ (reverse) primers were used. To normalize the expression level of CMP-SAT, the transcript level of Chinese hamster β-actin was analyzed using real-time PCR simultaneously. To analyze the transcript level of Chinese hamster β-actin, 5′-AGC TGA GAG GGA AAT TGT GCG-3′ (forward) and 5′-GCA

ACG GAA CCG CTC ATT-3′ (reverse) primers were used. Following real-time PCR, raw data were analyzed using iQ™5 Optical System software (Bio-Rad).

Measurement of the CMP-sialic acid level in CHO cells

Level of CMP-sialic acid in CHO cells was quantified as described previously (Tomiya et al. 2001). In brief, 1.0×10^7 cells were lysed in ice-cold 75% (v/v) ethanol using a sonic cell disruptor (Vibra Cell 130; Sonics & Materials). Soluble fractions were separated by 20,000 × g centrifugation for 10 min at 4°C and lyophilized. To stabilize CMP-sialic acid, samples were resuspended in 120 μL of 40 mM phosphate buffer, pH 9.2, and centrifuged. The resulting supernatant was filtered through a 10,000 molecular weight cut off membrane (Microcon®; Millipore, Bedford, MA). Sugar nucleotides were separated on a CarboPac PA-1 column (Dionex, Sunnyvale, CA) and detected at Abs_{260} by absorbance detector (Model 486 tuneable UV visible absorbance detector; Waters, Milford, MA). Intracellular CMP-sialic acid levels were normalized in relation to the cell number.

Production and purification of rhEPO

EC2-1H9 cells in an exponential growth phase, and the selected cell lines, were seeded (5.0×10^6 cells) in a T-175 cm^2 culture flask (Nunc) in MEM-α supplemented with 10% (v/v) dFBS, 3.5 g L^{-1} glucose, 1% (v/v) Ab-Am solution and 20 nM MTX. After 3 days, medium was replaced with serum-free medium (CHO-S-SFM II; Gibco), and culture was continued for 2 more days. Culture supernatant, containing rhEPO, was harvested and filtered through a membrane (pore-size 0.45 μm).

The filtered supernatant was concentrated, the buffer changed to PBS by ultrafiltration (AmiconUltra; Millipore), and rhEPO was purified by immunoaffinity chromatography on a column containing CNBr-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden), coupled with mouse anti-hEPO monoclonal antibodies (R&D Systems, Minneapolis, MN). Samples were loaded and washed with PBS, bound rhEPO was eluted with 0.1 M glycine/0.5 M NaCl, pH 2.8, and eluates were promptly neutralized with 1.0 M Tris–HCl, pH 9.0. Purified rhEPO was concentrated and dialyzed with distilled water by ultrafiltration (AmiconUltra; Millipore) and lyophilized following determination of concentration by Quant-iT™ protein assay kit (Invitrogen).

IEF analysis

Purified rhEPO (10 μg) was applied to Novex® Pre-Cast IEF gel, pH 3-7 (Invitrogen). Isoforms of rhEPO were separated at a constant 5 mA current for 2 h and focused at 500 V for 30 min. Isoform patterns were visualized by Coomassie Blue staining (Gel Code® Blue stain reagent; Pierce, Rockford, IL), and band intensities were analyzed using Multi Gauge version 3.0 software (GE Healthcare, Uppsala, Sweden).

Quantitative determination of sialic acid content in rhEPO

The sialic acid content of rhEPO was determined using the OPD-labeling method as described previously (Anumula 1995). In brief, sialic acid was detached from purified rhEPO in 0.5 M $NaHSO_4$ for 20 min at 80°C and derivatized with OPD–2HCl (Sigma) for 40 min at 80°C. OPD-labeled sialic acid was separated on a C_{18} reversed-phase column (Shim-pack CLC-ODS; Shimadzu, Kyoto, Japan) and detected by a fluorescence detector (Model 474; Waters) with wavelengths 230 nm emission and 425 nm excitation.

Analysis of sialylation profile of N-linked glycans from rhEPO

To release *N*-linked glycans, purified rhEPO (50 μg) was resuspended in *N*-glycanase reaction buffer (20 mM sodium phosphate, 0.02% sodium azide, pH 7.5) containing 0.1% SDS and 50 mM β-mercaptoethanol. rhEPO was denatured by boiling for 5 min and added with 0.75% NP-40. The mixture was incubated with 2 U of *N*-glycosidase F (Roche) overnight at 37°C to release *N*-linked glycans from rhEPO. Peptides and detergents were then removed using Glycoclean™ R cartridge, and salts were removed using Glycoclean™ H cartridge (Glyko®; ProZyme®, Hayward, CA). Purified *N*-linked glycans were derivatized with 2-AB using Signal™ labeling kit (Glyko®), and non-reacted 2-AB reagents were removed using Glycoclean™ S cartridge. The above procedures were performed following the manufacturer's instructions.

2-AB derivatized *N*-linked glycans from rhEPO were separated according to the number of sialic acids using an anion-exchange column (TSKgel DEAE-5PW, 7.5 mm × 75 mm; Tosoh, Tokyo, Japan). Eluents and gradient conditions

were as described previously (Llop et al. 2007). Samples were eluted over a 35 min linear gradient from 0% (v/v) to 100% (v/v) solvent A (solvent A: 50% 500 mM ammonium formate, pH 4.5. 30% water and 20% acetonitrile (ACN); solvent B: 80% water and 20% ACN) at a flow rate of 0.4 mL min^{-1} at 30°C and detected by a fluorescence detector (Model 474; Waters) at wavelengths 330 nm emission and 420 nm excitation. Number of sialic acid in each peak was determined by comparison with peaks in standard 2-AB bovine fetuin *N*-linked glycan library (Glyko®).

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Conflict of interest

None declared.

Abbreviations

α2,3-ST, α2,3-sialyltransferase; β1,4-GalT, β1,4-galactosyltransferase; 2-AB, 2-aminobenzamide; Ab-Am, antibiotic-antimycoti; ACN, acetonitrile; CHO, Chinese hamster ovary; CMP, cytidine monophosphate; dFBS, dialyzed fetal bovine serum; EPO, erythropoietin; Gal, galactose; GalT, galactosyltransferase; GlcNAc, *N*-acetyl glucosamine; GNE, UDP-GlcNAc 2-epimerase; hEPO, human erythropoietin; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; ManNAc, *N*-acetyl mannosamine; MEM, modified Eagle's medium; MNK, ManNAc kinase; MTX, methotrexate; NeuNAc, *N*-acetyl neuraminic acid (sialic acid); Ni-NTA, nickel-nitrilotriacetic acid; OPD, *o*-phenylenediamine; rhEPO, recombinant human erythropoietin; RT–PCR, reverse transcription–polymerase chain reaction; SAT, sialic acid transporter; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UDP, uridine diphosphate.

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