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Genetic engineering of CHO cells producing human interferon-γ by transfection of sialyltransferases

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Natural human interferon-γ (hIFN-γ) contains mainly biantennary complex-type sugar chains. We previously remodeled the branch structures of *N*-glycans on hIFN-γ in Chinese hamster ovary (CHO) cells by overexpressing UDP-*N*-acetylglucosamine: α1,6-D-mannoside β1,6-*N*-acetylglucosaminyltransferase (GnT-V). Normal CHO cells primarily produced hIFN-γ having biantennary sugar chains, whereas a CHO clone, designated IM4/Vh, transfected with GnT-V, primarily produced hIFN-γ having GlcNAcβ1-6 branched triantennary sugar chains when sialylation was incomplete and an increase in poly-*N*-acetyllactosamine (Galβ1-4GlcNAcβ1-3)n was observed. In the present study, we introduced mouse Galβ1-3/4GlcNAc-R α2,3-sialyltransferase (ST3Gal IV) and/or rat Galβ1-4GlcNAc-R α2,6-sialyltransferase (ST6Gal I) cDNAs into the IM4/Vh cells to increase the extent of sialylation and to examine the effect of sialyltransferase (ST) type on the linkage of sialic acid. Furthermore, we speculated that sialylation extent might affect the level of poly-*N*-acetyllactosamine. We isolated four clones expressing different levels of α2,3-ST and/or α2,6-ST. The extent of sialylation of hIFN-γ from the IM4/Vh clone was 61.2%, which increased to about 80% in every ST transfectant. The increase occurred regardless of the type of overexpressed ST, and the proportion of α2,3- and α2,6-sialic acid corresponded to the activity ratio of α2,3-ST to α2,6-ST. Furthermore, the proportion of *N*-glycans containing poly-*N*-acetyllactosamine was significantly reduced (less than 10%) in the ST transfectants compared with the parental IM4/Vh clone (22.9%). These results indicated that genetic engineering of STs is highly effective for regulating the terminal structures of sugar chains on recombinant proteins in CHO cells.

Keywords: **sialylation, sialyltransferase, CHO, poly-*N*-acetyllactosamine, interferon-γ**

Abbreviations: **hIFN-γ, human interferon-γ; CHO, Chinese hamster ovary; GnT-V, UDP-*N*-acetylglucosamine: α1,6-D-mannoside β1,6-*N*-acetylglucosaminyltransferase; α2,3-ST, α2,3-sialyltransferase; α2,6-ST, α2,6-sialyltransferase; iGnT, UDP-*N*-acetylglucosamine: *N*-acetyllactosaminide β1,3-*N*-acetylglucosaminyltransferase; β1,4-GalT, UDP-galactose: β-D-*N*-acetylglucosaminide β1,4-galactosyltransferase; TGN, *trans*-Golgi network; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetylneuraminic acid; PA-, pyridylamino-; HPLC, high-performance liquid chromatography.**

Introduction

Chinese hamster ovary (CHO) cells are widely used for production of recombinant glycoproteins for human therapy. Currently, genetic engineering of CHO cells is being refined to produce more effective recombinant glycoproteins. Most of such engineering of CHO cells has been conducted so that the glycosylation of recombinant glycoproteins is more akin to the native. Contrary, we have been attempting to engineer CHO cells, with the aim of producing useful glycoproteins with improved functions compared with natural one.

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We previously reported remodeling of sugar chains on hIFN-γ into multiantennary structures by overexpression of GnT-IV (UDP-*N*-acetylglucosamine: α1,3-D-mannoside β1,4-*N*-acetylglucosaminyltransferase) and/or GnT-V (UDP-*N*-acetylglucosamine: α1,6-D-mannoside β1,6-*N*-acetylglucosaminyltransferase) in CHO cells [1]. The purpose of the study was to demonstrate the effects of GnT-IV and GnT-V on *N*-glycan branching, however, during the study, we noticed important problems other than *N*-glycan branching. Those concerned undersialylation and poly-*N*-acetyllactosamine biosynthesis.

In the branch-remodeled sugar chains, it was expected that sialic acid content would increase along with the number of branches. However, the increase in sialic acid content was not as significant as expected because of incomplete sialylation. Santell *et al*. [2] also reported incomplete sialylation in a

number of glycoproteins produced by CHO cells, particularly under high-productivity conditions. Thus, sialylation seems to be incomplete in CHO cells. Incomplete sialylation causes a rapid clearance of glycoproteins from blood circulation via the recognition of exposed galactose by asialoglycoprotein receptor on hepatocytes [3,4]. Therefore, augmentation of sialylation was considered a necessary follow-up strategy for remodeling sugar chains in CHO cells in order to produce therapeutic glycoproteins with a longer circulatory half-life.

In addition, CHO-derived glycoproteins differ significantly from human-derived glycoproteins with regard to the type of sialic acid linkage. Natural human glycoproteins usually contain sialic acids in both α2,6- and α2,3-linkages [5], whereas CHO-derived glycoproteins have only α2,3-linked sialic acid [6–8], due to the lack of a functional copy of the gene for α2,6-sialyltransferase (α2,6-ST) in CHO cells [9]. In the present study, we attempted to genetically engineer CHO cells by transfection of sialyltransferases (STs) to augment sialylation and introduce sialic acids in α2,6-linkage as well as in α2,3-linkage.

Furthermore, we investigated the relationship between the sialylation and poly-*N*-acetyllactosamine (Galβ1-4GlcNAcβ1-3)n biosynthesis. We observed an increase in poly-*N*-acetyllactosamine in the above-mentioned branch-remodeled sugar chains of hIFN-γ. Poly-*N*-acetyllactosamine glycosylation is associated with GlcNAcβ1,6- branching in both *N*- and *O*-glycans, the rate-limiting step for their formation apparently being the degree of GlcNAcβ1,6-branching and not the activity of iGnT (UDP-*N*-acetylglucosamine: *N*-acetyllactosaminide β1,3-*N*-acetylglucosaminyltransferase). Otherwise stated, increased GnT-V, and not iGnT, activity results in increased formation of poly-*N*-acetyllactosamine chains [10]. Indeed, in *N*-glycans, poly-*N*-acetyllactosamines are added preferentially to triantennary and tetraantennary sugar chains containing a GlcNAcβ1,6-linkage formed by GnT-V [11–14]. This preferential addition occurs because β1,6-branching produces the preferred substrate for iGnT, which is responsible for the initiation and, together with β1,4-GalT (UDP-galactose: β-D-*N*-acetylglucosaminide β1,4-galactosyltransferase), elongation of poly-*N*-acetyllactosamines [15]. Such observations are in accordance with our results regarding the increase in poly-*N*-acetyllactosamine on hIFN-γ derived from a CHO clone overexpressing GnT-V [1]. Other factors that regulate the biosynthesis of poly-*N*-acetyllactosamines have also been reported, including the rate of Golgi transit of proteins [16–18] and branch specificities of iGnT and β1,4-GalT [19]. Furthermore, a competitive reaction between ST and iGnT was considered another possible factor because the two enzymes use the same terminal galactosyl residue (Galβ1-4GlcNAc) and they may be overlapping within the same Golgi cisternae. The overlapping localization of the two enzymes is possible since 1) ST has been localized in the *trans*-Golgi cisternae and *trans*-Golgi network (TGN) [20–23] and 2) iGnT must be located in a region capable of repeated interaction with β1,4-GalT that is also located in the *trans* Golgi cisternae and TGN [20–22, 24–26].

To assess the hyposialylation problem and possible competition between poly-*N*-acetyllactosamine formation and sialylation, we genetically modified CHO cells by transfection of two types of STs. We introduced mouse Galβ1-3/4GlcNAc-R α2,3-sialyltransferase (ST3Gal IV) and/or rat Galβ1-4GlcNAc-R α2,6-sialyltransferase (ST6Gal I) cDNAs into the previously reported CHO IM4/Vh clone, which overexpress GnT-V [1]. The IM4/Vh clone was a good material to test the augmentation of sialylation and its effect on the level of poly-*N*-acetyllactosamine. Here, we demonstrate an elevation of sialylation extent in the triantennary sugar chains of hIFN-γ by overexpressing STs in the IM4/Vh clone and confirm the competitive nature of α2,3- and α2,6-sialylation, regardless of the substrate specificity of the two enzymes, α2,3-ST and α2,6-ST. Furthermore, the effect of enhanced sialylation on the biosynthesis of poly-*N*-acetyllactosamine is discussed.

Materials and methods

Materials

GENETICIN (G418), CHO-S-SFM II medium, CD-CHO medium, and OPTI-MEM I medium were purchased from Life Technologies, Inc. Hygromycin B was purchased from Wako Pure Chemical Industries, Japan, methotrexate (MTX) was from Sigma, dialyzed fetal bovine serum (dFBS) was from JRH Biosciences, and glycopeptidase A (almond), β-galactosidase (jack bean), endo-β-galactosidase (*Escherichia freundii*), and FITC-SSA (*Sambucus sieboldiana*) were obtained from Seikagaku Kogyo, Japan. Sialidase (neuraminidase, *Arthrobacter ureafaciens*) was obtained from Nacalai Tesque, Japan, α-fucosidase (bovine kidney) was from Oxford Glycoscience, UK, and PA-sugar chain standards were obtained from Takara, Japan.

Culture of CHO cells

Experiments were performed using two hIFN-γ producing CHO cell lines, IM4 and IM4/Vh [1]. The IM4 clone is a subclone of the HIIF-D clone (obtained from American Type Culture Collection, No. CRL-8200) that stably produces a high level of hIFN-γ. The hygromycin-resistant IM4/Vh clone was established by overexpression of GnT-V in the IM4 clone. Cells were cultured at 37°C (5% CO_2/air) in tissue culture flasks containing CHO-S-SFM II medium and 10% dFBS supplemented with 250 nM MTX as described previously [1]. Hygromycin B (200 μg/ml) was added to the IM4/Vh clone culture. In addition to 200 μg/ml hygromycin B, G418 was also added at 300 μg/ml to ST transfected cell cultures. hIFN-γ was secreted into the culture media as described [1]. The culture supernatants were filtered (0.22 μm) and stored at −20°C.

Preparation of α2,3-ST and α2,6-ST expression vectors

Mouse α2,3-ST (ST3Gal IV in Tsuji *et al.* [27]) gene and rat α2,6-ST (ST6Gal I in Tsuji *et al.* [27]) gene were

isolated from Mouse Liver Marathon-Ready™ cDNA (CLONTECH) and Rat Liver cDNA Library (CLONTECH), respectively, by PCR based on previously reported nucleotide sequences [28,29]. Mouse ST3Gal IV and rat ST6Gal I genes were amplified using a PCR amplification kit (Takara) using the primers P-N1(*N*-terminal primer) and P-C1(*C*-terminal primer) for ST3Gal IV, and P-N2 and P-C2 for ST6Gal I. The primer sequences were as follows: P-N1, 5′-CCCGAATTCCCTGCTGCCTGCTGAGAAACATGAC-CAGCAAATCTCACTGGAAGC-3′; P-C1, 5′-GCTGAATT-CCAGGTCAGAAGTATGTGAGGTTCTTGAC-3′; P-N2, 5′-CCTCTCGAGCACAGTGGCTCTCCTGTCTGGACC-3′; P-C2, 5′-AAGCTCGAGGGTGCCTGGCTAGGTACTCAAC-AACG-3′. The reaction mixtures were subjected to 1% agarose gel electrophoresis and fragments of approximately 1040 bp for mouse ST3Gal IV and 1300 bp for rat ST6Gal I were recovered. The isolated PCR fragments were sequenced using an automatic DNA sequencer (ABI 373S, Perkin-Elmer Japan, Inc.). The fragments were cloned into the pCR2.1 vector (Invitrogen) by the TA cloning method using the Original TA Cloning Kit (Invitrogen). The resultant plasmids were designated pCR2.1-mα2,3-ST and pCR2.1-rα2,6-ST, respectively. The *Kpn*I/*Hind*III fragments of these plasmids were used to construct expression vectors.

The expression vector pCXN2Zb was constructed by inserting the lacZ coding region (*Eco*RI fragment) containing multicloning sites into the *Eco*RI site of the expression vector pCXN2 [30] (a generous gift from Dr. Jun-ichi Miyazaki, Osaka University, Japan), which contained a neomycin-resistance gene. An ST3Gal IV expression vector, pCXN2Zb-mα2,3-ST, and an ST6Gal I expression vector, pCXN2Zb-rα2,6-ST, were constructed by inserting the entire mouse ST3Gal IV or rat ST6Gal I coding regions (above described *Kpn*I/*Hind*III fragments), respectively, into the *Kpn*I/*Hind*III site of the expression vector pCXN2Zb. Another ST6Gal I expression vector pCXN2Zb(-Neo)-rα2,6-ST was constructed from the pCXN2Zb-rα2,6-ST as follows: the neomycin-resistance gene was excised by *Sal*I/*Ssp*I digestion and the plasmid was then blunt-ended (by T4 DNA polymerase, DNA Blunting Kit, Takara) and subsequently re-ligated.

Isolation of clones overexpressing α2,3-ST and/or α2,6-ST

The IM4/Vh clone was used as a parent. The ST3Gal IV or ST6Gal I expression vectors (pCXN2Zb-mα2,3-ST and pCXN2Zb-rα2,6-ST, respectively) were separately introduced into the IM4/Vh clone by electroporation. Near-confluent IM4/Vh cells were harvested, suspended in 0.4 ml of OPTI-MEM I medium, and subjected to electroporation as described [1]. After that, the cells were transferred to CHO-S-SFM II medium containing 10% dFBS, 250 nM MTX, and 200 μg/ml hygromycin B. The cells were then seeded on 100 mm tissue culture dishes at an appropriate dilution. Two days later, G418 (300 μg/ml) was added and the cells were cultured further. After approximately two weeks, G418-resistant cells were picked. Clones overexpressing α2,3-ST or α2,6-ST (IM4/Vh/3S and IM4/Vh/6S, respectively) were selected from the G418-resistant clones by measuring ST activities.

The IM4/Vh/3S clone, overexpressing α2,3-ST, was then transfected with pCXN2Zb(-Neo)-rα2,6-ST by electroporation as described above and the cells were transferred to CHO-S-SFM II medium containing 10% dFBS, 250 nM MTX, and 200 μg/ml hygromycin B. After three days, the cells were resuspended in PBS containing 0.1% BSA. FITC labeled SSA (*Sambucus sieboldiana*) was then added to a final concentration of 20 μg/ml. After a 30 min incubation on ice, cells were washed with PBS containing 0.1% BSA and sorted by a flow cytometer (EPICS ELITE, Coulter). Cells showing high fluorescence were sorted, amplified to 1×10^7 cells, and sorted again. Thus, the sorting was repeated four times. Subsequently, the enriched SSA-binding cells were cultured by limit dilution and single clones were isolated. From these clones, two showing moderate or high α2,6-ST activity (IM4/Vh/3S/6S-1 and IM4/Vh/3S/6S-2, respectively) were selected.

Glycosyltransferase activity assays

Assays of α2,3-ST, α2,6-ST, GnT-V, and iGnT activities were performed using pyridylaminated (PA-) sugar chains as substrates, and the reaction products were analyzed by reversed-phase HPLC. GnT-V activity was assayed using an 800 μM PA-agalacto biantennary sugar chain as a substrate, as described previously [1]. The iGnT assay was performed similarly, using PA-lacto-*N*-neotetraose as a substrate. The reaction mixture contained 10 mM HEPES buffer (pH 7.2), 80 mM UDP-GlcNAc, 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl, 200 mM GlcNAc, 5.6 mM γ-galactonolactone, 0.2% Triton X-100, and 800 μM PA-lacto-*N*-neotetraose. Assays of α2,3- and α2,6-ST were also performed using PA-lacto-*N*-neotetraose as a substrate. The reaction mixture contained 10 mM HEPES buffer (pH 7.2), 80 mM CMP-*N*-acetylneuramic acid (CMP-NeuAc), 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl, 20 μM 2,3-dehydro-2-deoxy-NeuAc, 5.6 mM γ-galactonolactone, 0.2% Triton X-100, and 800 μM PA-lacto-*N*-neotetraose. The activities were calculated based on the peak area of each product. The specific activities of GnT-V and iGnT were expressed as nmol and pmol of GlcNAc transferred per hour per 10^6 cells, respectively. The specific activities of α2,3-ST and α2,6-ST were expressed as nmol of NeuAc transferred per hour per 10^6 cells.

Purification of hIFN-γ, release of sugar chains, and pyridylamination

Recombinant hIFN-γ was purified by immunoaffinity chromatography and its sugar chains released and pyridylaminated as described [1]. Briefly, purified hIFN-γ was digested with modified trypsin (Promega) and glycopeptides were purified by gel filtration (Sephadex G-25, Amersham Pharmacia

Biotech). The glycopeptides were digested with glycopeptidase A (from almond) and released sugar chains were obtained by treating the digested products with Sep-Pak Plus C18 Cartridges (Waters). The sugar chains were pyridylaminated by the method of Kuraya and Hase [31]. Excess reagents were removed by gel filtration on a Sephadex G-15 (Amersham Pharmacia Biotech) column.

Structural analysis of PA-sugar chains

PA-sugar chains were initially analyzed by anion-exchange HPLC and the sialylation pattern was analyzed on a Mono Q column (5×50 mm, Amersham Pharmacia Biotech) as described [1]. Next, whole PA-sugar chains were desialylated with sialidase from *Arthrobacter ureafaciens* in 0.2 M ammonium acetate buffer, pH 5.0, for 20 h at 37°C. The desialylated sugar chains were separated as a neutral fraction by anion-exchange HPLC.

Sugar chains containing poly-*N*-acetyllactosamines were analyzed by measuring peaks that disappeared after endo-β-galactosidase digestion. For this, desialylated PA-sugar chains from each hIFN-γ were first digested with β-galactosidase from jack bean, in 0.1 M citrate-phosphate buffer, pH 4.0, for 20 h at 37°C. The digested PA-sugar chains were further treated with endo-β-galactosidase from *Escherichia freundii*, in 0.1 M citrate-phosphate buffer, pH 5.0, for 20 h at 37°C. The products before and after digestion by endo-β-galactosidase were analyzed by reversed-phase HPLC equipped with a Shim-pack CLC-ODS column (6×150 mm, Shimadzu, Japan). Elution was performed at a flow rate of 0.8 ml/min at 55°C. The column was equilibrated with a mixture of solvent A (10 mM sodium phosphate buffer, pH 3.8) and B (10 mM sodium phosphate buffer, 0.5% l-butanol, pH 3.8), 90:10 by volume. The ratio of solvent B was increased to A:B = 35:65 over 90 min by a linear gradient. PA-sugar chains were detected by fluorescence at excitation and emission wavelengths of 320 nm and 400 nm, respectively.

To analyze the branch structures of sugar chains, desialylated PA-sugar chains were simultaneously treated with β-galactosidase (jack bean), endo-β-galactosidase (*Escherichia freundii*), and α-fucosidase (bovine kidney). Obtained backbone sugar chains were separated and quantitated by reversed-phase HPLC equipped with a Shim-pack CLC-ODS column, as described above.

Measurement of the extent of sialylation and determination of sialic acid linkage

Sugar chains released from hIFN-γ were purified on cellulose cartridges (Takara) according to the manufacturer's protocol. The extent of sialylation was determined by measuring the molar ratio of sialic acid to penultimate galactose. For this, the purified (non-pyridylaminated) sugar chains were digested with sialidase (*Arthrobacter ureafaciens*) in 0.2 M ammonium acetate buffer, pH 5.0, for 20 h at 37°C, then further digested with β-galactosidase (jack bean) in 0.1 M citrate-phosphate buffer, pH 4.0 for 20 h at 37°C. To remove proteins from the reaction mixture, the mixture was passed through a Microcon SCX (Millipore). The amount of sialic acid and galactose in the resultant solution was analyzed by HPAEC-PAD (high-pH anion-exchange chromatography with pulsed amperometric detection) using the Dionex Bio-LC system equipped with a CarboPac PA1 column (4×250 mm; Dionex) and a CarboPac PA1 guard column (4×50 mm; Dionex). Sialic acid was eluted by 100 mM NaOH containing 100 mM CH_3COONa, at a flow rate of 1.0 ml/min. Galactose was eluted by 25 mM NaOH at a flow rate of 1.0 ml/min. Monosaccharides were detected using a pulsed electrochemical detector (PED) under integrated pulsed amperometry.

Sialic acid linkage was determined by using linkage-sensitive sialidases. The purified sugar chains were digested with (a) *A. ureafaciens* sialidase, which cleaves both α2,3- and α2,6-linked sialic acids, in 0.2 M ammonium acetate buffer, pH 5.0; or (b) NANase I (GLYKO), which selectively cleaves α2,3-linked sialic acids, in 50 mM sodium phosphate buffer, pH 6.0. The amount of released sialic acids in the reaction mixture was analyzed by HPAEC-PAD, as described above. The molar ratio of α2,3- to α2,6-linked sialic acids was estimated from the two values.

Results

Isolation of clones overexpressing α2,3-ST and/or α2,6-ST

The IM4/Vh clone was transfected with either mouse α2,3-ST or rat α2,6-ST, designated, according to the nomenclature of Tsuji *et al*. [27], ST3Gal IV and ST6Gal I, respectively, using the expression vectors containing a neomycin-resistance gene. Thirty clones each, which acquired G418-resistance, were isolated from cells transfected with the ST3Gal IV gene and from those transfected with the ST6Gal I gene. From these clones, those overexpressing the ST gene, but showing no difference in β1,4-GalT activity, GnT-V activity or hIFN-γ productivity compared to the parental IM4/Vh clone, were selected. From each group of the above thirty clones, one clone showing high α2,3-ST activity and one clone showing high α2,6-ST activity were selected and designated IM4/Vh/3S and IM4/Vh/6S, respectively. The α2,3-ST, α2,6-ST, GnT-V and iGnT activities in each clone are summarized in Table 1. α2,6-ST activity was not detected in IM4/Vh and IM4/Vh/3S clones because normal CHO cells do not contain α2,6-ST [9]. α2,3-ST activity of the IM4/Vh/3S clone was approximately nine-fold higher than that of the IM4/Vh clone. iGnT activity did not differ significantly between the two clones and the parental IM4/Vh clone.

The IM4/Vh/3S clone was further transfected with ST6Gal I. Before selecting clones overexpressing α2,6-ST and α2,3-ST, we attempted to enrich the cells expressing α2,6-sialic acid by detecting them with FITC-SSA, which recognizes α2,6-sialic acid. Cells showing SSA-binding were sorted using a flow cytometer. By repeating the sorting,

Table 1. Glycosyltransferase activities in each CHO clone. Activities of α2,3-ST, α2,6-ST, and iGnT were assayed using PA-lacto-*N*-neotetraose as a substrate. GnT-V activity was assayed using PA-agalacto biantennary sugar chain as a substrate. The specific activities of α2,3-ST and α2,6-ST are expressed as nmol of NeuAc transferred per hour per 10^6 cells. The specific activities of GnT-V and iGnT are expressed as nmol and pmol of GlcNAc transferred per hour per 10^6 cells, respectively. Values are the means ± S.D. of duplicate assays. ND, not detected

Clone	*α2,3-ST*	*α2,6-ST*	*GnT-V*	*iGnT*
	(nmol/hour/10^6cells)			(pmol/hour/10^6cells)
IM4	0.06 ± 0.00	ND	0.46 ± 0.02	1.53 ± 0.10
IM4/Vh	0.06 ± 0.00	ND	7.74 ± 0.52	1.66 ± 0.05
IM4/Vh/3S	0.52 ± 0.01	ND	6.60 ± 0.47	1.59 ± 0.05
IM4/Vh/6S	0.07 ± 0.00	5.61 ± 0.42	6.46 ± 0.62	1.78 ± 0.23
IM4/Vh/3S/6S-1	0.45 ± 0.04	0.24 ± 0.02	5.84 ± 0.83	1.93 ± 0.08
IM4/Vh/3S/6S-2	0.49 ± 0.04	9.74 ± 0.37	5.58 ± 0.73	1.56 ± 0.14

SSA-binding clones were enriched. Among these clones, two showing moderate or high level of α2,6-ST activity (IM4/Vh/3S/6S-1 and IM4/Vh/3S/6S-2, respectively) were selected. Activities of α2,3-ST, GnT-V and iGnT in the two clones were not altered compared with the IM4/Vh/3S clone.

It is noteworthy that α2,3-ST reacted more slowly than α2,6-ST with PA-lacto-*N*-neotetraose, which we used as a substrate for the enzyme activity assays. To our knowledge, this phenomenon has not been reported, however, when we measured the activity of equal amounts (based on units defined by manufacturers) of commercially available α2,3-ST (from rat, CALBIOCHEM) and α2,6-ST (from rat, CALBIOCHEM) by our assay method, α2,6-ST showed 9.2-fold higher activity than α2,3-ST toward PA-lacto-*N*-neotetraose.

The extent of sialylation and sugar chain structures of hIFN-γ in each clone

hIFN-γ produced by each CHO clone were purified and the sugar chains at the two glycosylation sites of hIFN-γ were then collectively excised and purified. The extent of sialylation of hIFN-γ was determined by measuring the molar ratio of sialic acid to penultimate galactose by sequential exoglycosidase treatment (Table 2). Average number of sialic acid residues per sugar chain was calculated from the proportion of asialo, monosialo, disialo, trisialo, and tetrasialo sugar chains based on anion-exchange HPLC analysis (Figure 1). In the hIFN-γ from the IM4 clone, on which sugar chains are biantennary [1], the extent of sialylation was 51.6% and the average number of sialic acid residues per sugar chain was 1.33. In the hIFN-γ from the

Table 2. Structural analysis of sugar chains on hIFN-γ produced by each CHO clone. The extent of sialylation was determined by measuring the molar ratio of sialic acid to galactose. The total amount of sugar chains containing poly-*N*-acetyllactosamine was determined by measuring peak areas that are indicated by arrows in Figure 3. Branch structures of sugar chains were determined by analyzing backbone structures obtained by removal of the side chains as described in Materials and methods. ND, not detected

	Clone					
	IM4 (%)	*IM4/Vh (%)*	*IM4/Vh/3S (%)*	*IM4/Vh/6S (%)*	*IM4/Vh/3S/6S-1 (%)*	*IM4/Vh/3S/6S-2 (%)*
Extent of sialylation	51.6	61.2	80.8	79.8	81.2	86.4
Percentage of α2,3-linked sialic acid	100	100	100	17.4	79.3	26.4
Percentage of α2,6-linked sialic acid	0	0	0	82.6	20.7	73.6
Total amount of sugar chains containing poly-*N*-acetyllactosamine	5.9	22.9	6.2	6.1	9.2	4.0
Branch structure of sugar chains						
Biantennary	65.6	15.2	17.4	15.5	13.4	15.0
Triantennary (GlcNAcβ1,4-branched)	ND	ND	ND	ND	ND	ND
Triantennary (GlcNAcβ1,6-branched)	13.8	59.4	62.1	60.5	62.8	62.9
Tetraantennary	1.0	9.3	9.8	10.5	14.5	12.1

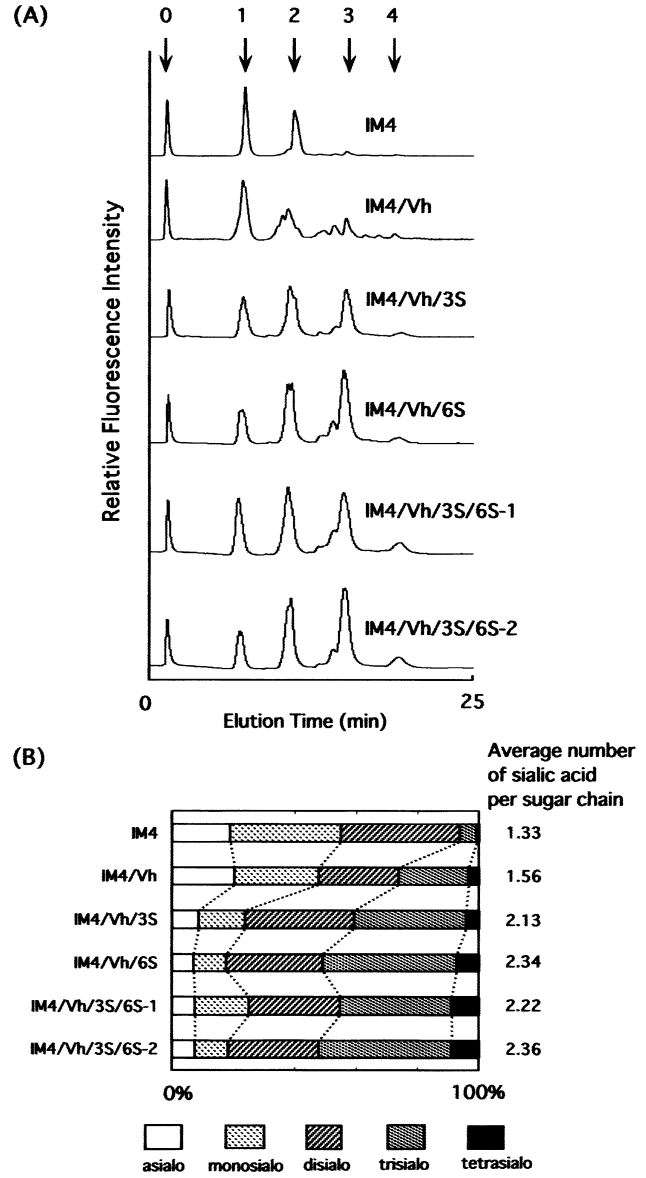

Figure 1. Sialylation patterns of hIFN-γ produced by each CHO clone. (A) Anion-exchange HPLC chromatograms of PA-sugar chains derived from hIFN-γ from different cell line. The PA-sugar chains were separated according to the negative charges. Arrows indicate the elution positions of asialo PA-sugar chains (0), monosialo PA-sugar chains (1), disialo PA-sugar chains (2), trisialo PA-sugar chains (3), and tetrasialo PA-sugar chains (4). The proportion of each fraction is shown in panel (B).

IM4/Vh clone, which is capable of the formation of triantennary sugar chains, the content of sialic acid was expected to increase along with the increase in the number of branches. However, the average number of sialic acid residues per sugar chain was only 1.56 (extent of sialylation: 61.2%). In contrast, sialylation was significantly augmented in the ST transfectants. We confirmed that the proportion of asialo, monosialo, disialo, trisialo, and tetrasialo sugar chains changed, showing a higher sialylation pattern in the ST transfectants (Figure 1). The average number of sialic acid residues per sugar chain increased to 2.13, 2.34, 2.22 and 2.36 in the IM4/Vh/3S, IM4/Vh/6S, IM4/Vh/3S/6S-1, and IM4/Vh/3S/6S-2 clones, respectively. The extent of sialylation in these clones increased to 80.8% (IM4/Vh/3S), 79.8% (IM4/Vh/6S), 81.2% (IM4/Vh/3S/6S-1), and 86.4% (IM4/Vh/3S/6S-2). However, it was striking that sialylation was still not complete in these ST transfectants.

The branching of the glycans on hIFN-γ from each clone was also analyzed in order to confirm that they were not altered by ST overexpression. As shown in Table 2, the percentage of bi-, tri-, and tetraantennary *N*-glycans was not different between either the ST transfectants and the parental IM4/Vh line or between clones (i.e., in all cases triantennary structures accounted for around 60% of the glycans). However, as previously described [1], hIFN-γ produced in the 'grandparental' IM4 clone carried mainly biantennary, and only 13.8% triantennary, structures.

Sialic acid linkage

The sialic acid linkage was determined (Table 2) by using linkage-sensitive sialidases. The sialic acid in hIFN-γ from the IM4/Vh/3S clone exclusively displayed α2,3-linkage, as expected. Conversely, α2,6-ST transfectants exhibited sialic acid in both α2,3- and α2,6-linkages. The proportion of sialic acid in α2,3- and α2,6-linkage was 17.4%:82.6% in IM4/Vh/6S, 79.3%:20.7% in IM4/Vh/3S/6S-1, and 26.4%:73.6% in IM4/Vh/3S/6S-2. The proportion of α2,3- and α2,6-sialic acid corresponded to the ratio of α2,3- to α2,6-ST activities (Figure 2).

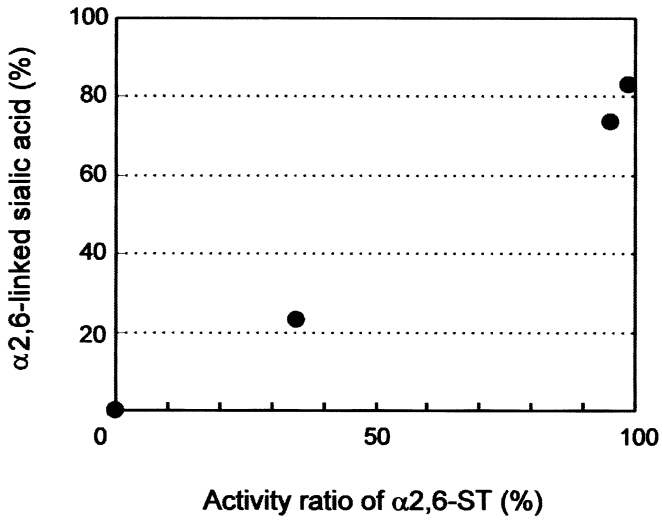

Figure 2. Relationship between the activity ratio of STs and the linkage of sialic acid. The percentage of α2,6-linked sialic acid ($=\alpha$2,6-linked sialic acid/[α2,6-linked sialic acid + α2,3-linked sialic acid] $\times$ 100) was plotted against the activity ratio of α2,6-ST ($=\alpha$2,6-ST activity/[α2,6-ST activity + α2,3-ST activity] $\times$ 100).

Content of sugar chains having poly-*N*-acetyllactosamine

The content of sugar chains having poly-*N*-acetyllactosamine was determined by the disappearance of HPLC peaks after endo-β-galactosidase digestion. The sugar chains were first treated with sialidase and then with β-galactosidase. The products before and after digestion by endo-β-galactosidase were analyzed by reversed-phase HPLC and profiles were compared (Figure 3, Table 2). The peaks that were absent after endo-β-galactosidase digestion were presumed to contain poly-*N*-acetyllactosamine structures. Digestion of hIFN-γ sugar chains from the IM4/Vh clone by endo-β-galactosidase eliminated several peaks. The total amount of sugar chains containing poly-*N*-acetyllactosamine was estimated as 22.9% of the overall sugar chains. This amount was significantly higher than observed for sugar chains from the IM4 clone. On the other hand, significantly fewer peaks were observed for the hIFN-γs from ST transfectants: 6.2%, 6.1%, 9.2%, and 4.0% in the IM4/Vh/3S, IM4/Vh/6S, IM4/Vh/3S/6S-1, and IM4/Vh/3S/6S-2 clones, respectively. Levels of poly-*N*-acetyllactosamine therefore decreased as the extent of sialylation increased (Figure 4).

Discussion

CHO-derived glycoproteins have only α2,3-linked sialic acid [6–8], whereas natural human glycoproteins usually contain sialic acids in both α2,6- and α2,3-linkages [5]. Recent advances in glycosylation analysis have enabled us to compare the sugar chain structures of recombinant glycoproteins with their natural human counterparts. Hence, the glycosylation profile of glycoproteins for use as pharmaceuticals must be fully characterized, and if possible, controlled. Furthermore, remodeling the glycosylation profile may serve to change *in vivo* bioactivity or pharmacokinetics of glycoproteins. As a result, genetic engineering of CHO cells is widely studied. In order to increase sialylation, Weikert *et al*. [32] overexpressed α2,3-ST in two CHO cells producing different glycoproteins and demonstrated an increase in clearance time of the glycoproteins. On the other hand, several groups have introduced α2,6-ST into recombinant CHO cells producing human glycoproteins in an attempt to produce more naturally glycosylated recombinant glycoproteins [33–37]. Although the significance of co-existing α2,6- and α2,3-linkages in sialic acid is not understood, it is desirable to produce recombinant glycoproteins with a glycosylation as close as possible to that of the native protein. Thus, in spite of the wide application of CHO cells for production of recombinant glycoproteins, the cells still require additional engineering, especially to modify sialylation.

In the present study, CHO cells have been engineered with respect to their sialylation performance because undersialylation was observed in the hIFN-γ we produced during an attempt to remodel sugar chains using CHO cells. We thought

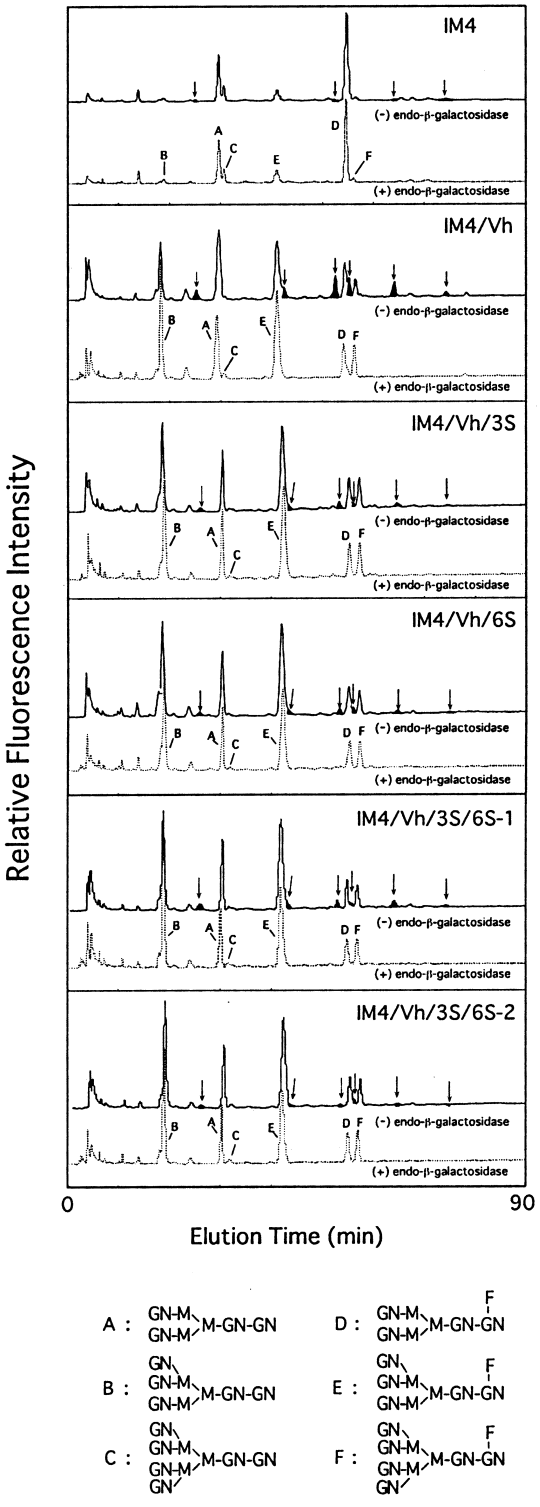

Figure 3. Reversed-phase HPLC of PA-sugar chains derived from hIFN-γ produced by each CHO clone. PA-sugar chains from each hIFN-γ were digested with sialidase and β-galactosidase. The digested PA-sugar chains were then treated with endo-β-galactosidase. The samples before and after endo-β-galactosidase digestion were analyzed by reversed-phase HPLC. Peaks that were absent after endo-β-galactosidase digestion are indicated by arrows.

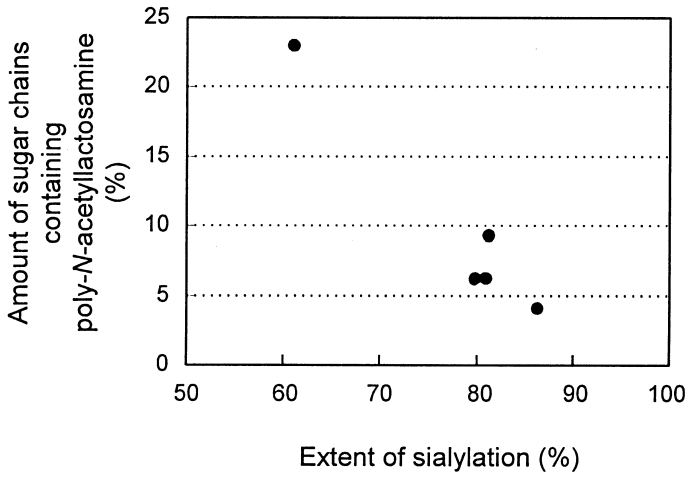

Figure 4. Relationship between the extent of sialylation and the level of poly-*N*-acetyllactosamine.

that more attention should be paid to undersialylation in CHO cells, while previous reports regarding genetic engineering of CHO cells mostly focused on giving sialic acid in both α2,6- and α2,3-linkages, as described by Lee *et al*. [33], Minch *et al*. [34], Zhang *et al*. [35], Bragonzi *et al*. [36], and Monaco *et al*. [37]. Engineering CHO cells to increase sialylation would be more necessary for biotechnology industry. In the hIFN-γ with biantennary sugar chains, produced by the IM4 clone, the extent of sialylation was 51.6% and the average number of sialic acid residues per sugar chain was 1.33. In hIFN-γ with triantennary sugar chains, produced by a GnT-V-transfectant (IM4/Vh clone), the number of sites where sialic acid could attach increased. However, sialylation did not increase (average number of sialic acid residues per sugar chain: 1.56, extent of sialylation: 61.2%) in proportion to the increase in the number of branches. In order to increase the extent of sialylation, we first introduced either α2,3-ST or α2,6-ST into the IM4/Vh clone. In both the IM4/Vh/3S and IM4/Vh/6S clones, sialylation extent increased, although only to about 80% of maximum. The results suggested the presence of other factors limiting sialylation.

We suspected that enzyme specificity toward *N*-glycan branches might be one of the factors. Previous reports have described the branch specificity of α2,3- and α2,6-STs. The Galβ1-4GlcNAcβ1-6Manα1-6 branch is extremely resistant to α2,6-sialylation. The α2,6-linked sialic acid is predominant on biantennary oligosaccharides [38], while α2,3-linked sialic acid is more frequently encountered on tri- and tetra-branched glycans [39]. Thus, the two types of sialyltransferases are believed to work with complementary specificity toward *N*-glycan branches. Therefore, we expected that overexpression of both α2,3- and α2,6-STs might improve the sialylation level, owing to their complementary actions. However, even in the double-ST transfectants, IM4/Vh/3S/6S-1 and IM4/Vh/3S/6S-2, overall sialylation extent was not greatly improved, although the IM4/Vh/3S/6S-2 clone showed about 5% greater sialylation than IM4/Vh/3S and IM4/Vh/6S. Thus, the complementary roles of α2,3- and α2,6-STs did not appear to improve the sialylation extent. The *in vitro* specificity appeared not to correlate with the branch-specific sialylation *in vivo*, judging from the finding that the proportion of α2,3- and α2,6-sialic acids simply corresponded to the ratio of α2,3- to α2,6-STs. Therefore, other factors, in addition to the level of ST expression, should be considered when investigating factors limiting the extent of sialylation. Other possible factors, including the transport of CMP-sialic acid into the Golgi apparatus, biosynthesis of CMP-sialic acid or the presence of sialidase in the cells, may be involved. Although several studies [32–37] have been reported regarding improvement of sialylation performance in CHO cells, the limitation of sialylation has not been addressed. Contrary, we showed this important issue through the quantitative analysis of sialylation. Further clarification and investigation of the factors limiting sialylation is required.

Furthermore, to be more interesting, we also found that augmentation of sialylation suppresses poly-*N*-acetyllactosamine biosynthesis. By overexpressing GnT-V, CHO cells (IM4/Vh) produced hIFN-γ containing triantennary sugar chains concomitantly with a poly-*N*-acetyllactosamine increase. Conversely, when STs were further overexpressed, the level of poly-*N*-acetyllactosamine was reduced significantly with no change in the branch structure. As described above, the extent of sialylation was increased by ST overexpression and the levels of poly-*N*-acetyllactosamine decreased with sialylation extent. Therefore, the capping of terminal galactosyl residues by sialic acid conceivably suppressed the formation of poly-*N*-acetyllactosamine. ST has been localized in the *trans*-Golgi and TGN [20–23]. Although the localization of iGnT is unclear, the enzyme must be located within the same region as β1,4-GalT, because poly-*N*-acetyllactosamine biosynthesis requires repeated reaction of iGnT and β1,4-GalT. β1,4-GalT has been localized to the *trans*-Golgi and TGN [20–22, 24–26]. Therefore, iGnT is potentially located in the same region as ST in the Golgi complex.

Poly-*N*-acetyllactosamine has been shown to increase on biantennary sugar chains in sialylation-incapable Lec2 CHO cells [40], also indicative of a competition between ST and iGnT. This observation supports our results. However, this phenomenon has not been reported in detail. In the present report, we augmented sialylation in CHO cells overexpressing GnT-V. This produced hIFN-γ containing GlcNAcβ1-6 branched triantennary sugar chains, and increased the levels of poly-*N*-acetyllactosamine. By using the hIFN-γ containing branch-remodeled sugar chains, we clearly showed the effects of augmenting sialylation on the biosynthesis of poly-*N*-acetyllactosamine. The IM4/Vh/3S and IM4/Vh/6S clones differed in sialic acid linkage type, but showed nearly identical extent of sialylation and levels of poly-*N*-acetyllactosamine. Similarly both clones of the doubly-transfected ST line had lower poly-*N*-acetyllactosamine than the parental IM4/Vh clone. Therefore, the suppression of poly-*N*-acetyllactosamine

biosynthesis by augmented sialylation seemed to occur regardless of sialic acid linkage type. Although α2,6-sialylation does not directly cap the C-3 position of terminal galactose, on which iGnT acts, α2,6-sialylated terminal galactose may be a poor substrate for iGnT. Similar situation occurs in the case of competition between α1,3-galactosyltransferase and α1,2-fucosyltransfearse, as reported in an attempt to reduce Galα1,3-Gal (α-galactosyl epitope) [41,42].

In conclusion, we have shown the augmentation of sialylation in CHO cells by overexpressing two types of STs. We found that the activity ratio of α2,3- to α2,6-ST directly affected the proportion of α2,3- and α2,6-sialic acid. A CHO clone producing the desired proportion of α2,3- and α2,6-sialic acid, which would produce more naturally glycosylated proteins, can theoretically be obtained by altering the activity ratio of α2,3- to α2,6-ST. However, even in the ST transfectants, sialylation was still incomplete. Augmentation of sialylation in CHO cells is important for the biotechnology industry. Therefore, other approaches to attaining complete sialylation must be tested. Furthermore, we showed that enhanced sialylation suppresses biosynthesis of poly-*N*-acetyllactosamine. These results indicate that augmentation of sialylation reduces heterogeneity in terminal structures of *N*-glycans by both capping the *N*-glycan terminal more consistently and suppressing the elongation of poly-*N*-acetyllactosamine. If the sialylation extent could be maximized, the level of poly-*N*-acetyllactosamine might be reduced even further. Our study has presented new data on the interaction of glycosyltransferases with recombinant glycoproteins produced by CHO cells. Such information will be useful for the construction of a more ideal host cell. Future studies are also planned to examine whether the forms of hIFN-γ produced in the present study have improved pharmacokinetic properties.

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References

1 Fukuta K, Abe R, Yokomatsu T, Kono N, Asanagi M, Omae F, Minowa MT, Takeuchi M, Makino T, Remodeling of sugar chain structures of human interferon-γ, *Glycobiology* **10**, 421–30 (2000).

2 Santell L, Ryll T, Etcheverry T, Santoris M, Dutina G, Wang A, Gunson J, Warner TG, Aberrant metabolic sialylation of recombinant proteins expressed in Chinese hamster ovary cells in high productivity cultures, *Biochem Biophys Res Commun* **258**, 132–7 (1999).

3 Ashwell G, Harford J, Carbohydrate-specific receptors of the liver, *Annu Rev Biochem* **51**, 531–54 (1982).

4 Cole ES, Nichols EH, Poisson L, Harnois ML, Livingston DJ, *In vivo* clearance of tissue plasminogen-activator – the complex role of sites of glycosylation and level of sialylation, *Fibrinolysis* **7**, 15–22 (1993).

5 Takeuchi M, Takasaki S, Miyazaki H, Kato T, Hoshi S, Kochibe N, Kobata A, Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells, *J Biol Chem* **263**, 3657–63 (1988).

6 Bergwerff AA, van Oostrum J, Asselbergs FA, Burgi R, Hokke CH, Kamerling JP, Vliegenthart JF, Primary structure of *N*-linked carbohydrate chains of a human chimeric plasminogen activator K2tu-PA expressed in Chinese hamster ovary cells, *Eur J Biochem* **212**, 639–56 (1993).

7 Conradt HS, Egge H, Peter-Katalinic J, Reiser W, Siklosi T, Schaper K, Structure of the carbohydrate moiety of human interferon-β secreted by a recombinant Chinese hamster ovary cell line, *J Biol Chem* **262**, 14600–5 (1987).

8 Watson E, Bhide A, van Halbeek H, Structure determination of the intact major sialylated oligosaccharide chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells, *Glycobiology* **4**, 227–37 (1994).

9 Svensson EC, Soreghan B, Paulson JC, Organization of the β-galactoside α2,6-sialyltransferase gene. Evidence for the transcriptional regulation of terminal glycosylation, *J Biol Chem* **265**, 20863–8 (1990).

10 Yousefi S, Higgins E, Daoling Z, Pollex-Kruger A, Hindsgaul O, Dennis JW, Increased UDP-GlcNAc: Galβ1-3GalNAc-R (GlcNAc to GalNAc) β-1,6-*N*-acetylglucosaminyltransferase activity in metastatic murine tumor cell lines. Control of polylactosamine synthesis, *J Biol Chem* **266**, 1772–82 (1991).

11 Sasaki H, Bothner B, Dell A, Fukuda M, Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA, *J Biol Chem* **262**, 12059–76 (1987).

12 Yamashita K, Ohkura T, Tachibana Y, Takasaki S, Kobata A, Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis, *J Biol Chem* **259**, 10834–40 (1984).

13 Yoshima H, Matsumoto A, Mizuochi T, Kawasaki T, Kobata A, Comparative study of the carbohydrate moieties of rat and human plasma α1-acid glycoproteins, *J Biol Chem* **256**, 8476–84 (1981).

14 Pierce M, Arango J, Rous sarcoma virus-transformed baby hamster kidney cells express higher levels of asparagine-linked tri- and tetraantennary glycopeptides containing [GlcNAc-β(1,6)Man-α(1,6)Man] and poly-*N*-acetyllactosamine sequences than baby hamster kidney cells, *J Biol Chem* **261**, 10772–7 (1986).

15 van den Eijnden DH, Koenderman AHL, Schiphorst WECM, Biosynthesis of blood group i-active polylactosaminoglycans. Partial purification and properties of an UDP-GlcNAc: *N*-acetyllactosaminide $\beta 1 \rightarrow 3$-*N*-acetylglucosaminyltransferase from Novikoff tumor cell ascites fluid, *J Biol Chem* **263**, 12461–71 (1988).

16 Nabi IR, Dennis JW, The extent of polylactosamine glycosylation of MDCK LAMP-2 is determined by its Golgi residence time, *Glycobiology* **8**, 947–53 (1998).

17 Nabi IR, Rodriguez-Boulan E, Increased LAMP-2 polylactosamine glycosylation is associated with its slower Golgi transit

during establishment of a polarized MDCK epithelial monolayer, *Mol Biol Cell* **4**, 627–35 (1993).
18 Wang W-C, Lee N, Aoki D, Fukuda MN, Fukuda M, The poly-*N*-acetyllactosamines attached to lysosomal membrane glycoproteins are increased by the prolonged association with the Golgi complex, *J Biol Chem* **266**, 23185–90 (1991).
19 Ujita M, McAuliffe J, Hindsgaul O, Sasaki K, Fukuda MN, Fukuda M, Poly-*N*-acetyllactosamine synthesis in branched *N*-glycans is controlled by complemental branch specificity of i-extension enzyme and β1,4-galactosyltransferase I, *J Biol Chem* **274**, 16717–26 (1999).
20 Berger EG, Hesford FJ, Localization of galactosyl- and sialyltransferase by immunofluorescence: evidence for different sites, *Proc Natl Acad Sci USA* **82**, 4736–9 (1985).
21 Rabouille C, Hui N, Hunte F, Kieckbusch R, Berger EG, Warren G, Nilsson T, Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides, *J Cell Sci* **108**, 1617–27 (1995).
22 Roth J, Subcellular organization of glycosylation in mammalian cells, *Biochim Biophys Acta* **906**, 405–36 (1987).
23 Roth J, Taatjes DJ, Lucocq JM, Weinstein J, Paulson JC, Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation, *Cell* **43**, 287–95 (1985).
24 Berger EG, Mandel T, Schilt U, Immunohistochemical localization of galactosyltransferase in human fibroblasts and Hela cells, *J Histochem Cytochem* **29**, 364–70 (1981).
25 Nilsson T, Pypaert M, Hoe MH, Slusarewicz P, Berger EG, Warren G, Overlapping distribution of two glycosyltransferases in the Golgi apparatus of Hela cells, *J Cell Biol* **120**, 5–13 (1993).
26 Roth J, Berger EG, Immunocytochemical localization of galactosyltransferase in Hela cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae, *J Cell Biol* **92**, 223–9 (1982).
27 Tsuji S, Datta AK, Paulson JC, Systematic nomenclature for sialyltransferases, *Glycobiology* **6**, 7:v–vii (1996).
28 Kono M, Ohyama Y, Lee Y-C, Hamamoto T, Kojima N, Tsuji S, Mouse β-galactoside α2,3-sialyltransferases: comparison of *in vitro* substrate specificities and tissue specific expression, *Glycobiology* **7**, 469–79 (1997).
29 Weinstein J, Lee EU, McEntee K, Lai P-H, Paulson JC, Primary structure of β-galactoside α2,6-sialyltransferase. Conversion of membrane-bound enzyme to soluble forms by cleavage of the NH_2-terminal signal anchor, *J Biol Chem* **262**, 17735–43 (1987).
30 Niwa H, Yamamura K, Miyazaki J, Efficient selection for high-expression transfectants with a novel eukaryotic vector, *Gene* **108**, 193–9 (1991).
31 Kuraya N, Hase S, Release of *O*-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions, *J Biochem* **112**, 122–6 (1992).
32 Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M, Lofgren J, Mehta S, Chisholm V, Modi N, Eppler S, Carroll K, Chamow S, Peers D, Berman P, Krummen L, Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins, *Nature Biotechnol* **17**, 1116–21 (1999).
33 Lee EU, Roth J, Paulson JC, Alteration of terminal glycosylation sequences on *N*-linked oligosaccharides of Chinese hamster ovary cells by expression of β-galactoside α2,6-sialyltransferase, *J Biol Chem* **264**, 13848–55 (1989).
34 Minch SL, Kallio PT, Bailey JE, Tissue plasminogen activator coexpressed in Chinese hamster ovary cells with α(2,6)-sialyltransferase contains NeuAc α(2,6)Gal β(1,4)Glc-N-AcR linkages, *Biotechnol Prog* **11**, 348–51 (1995).
35 Zhang X, Lok SHL, Kon OL, Stable expression of human α-2,6-sialyltransferase in Chinese hamster ovary cells: functional consequences for human erythropoietin expression and bioactivity, *Biochim Biophys Acta* **1425**, 441–52 (1998).
36 Bragonzi A, Distefano G, Buckberry LD, Acerbis G, Foglieni C, Lamotte D, Campi G, Marc A, Soria MR, Jenkins N, Monaco L, A new Chinese hamster ovary cell line expressing α2,6-sialyltransferase used as universal host for the production of human-like sialylated recombinant glycoproteins, *Biochim Biophys Acta* **1474**, 273–82 (2000).
37 Monaco L, Marc A, Eon-Duval A, Acerbis G, Distefano G, Lammotte D, Engasser J-M, Soria M, Jenkins N, Genetic engineering of α2,6-sialyltransferase in recombinant CHO cells and its effects on the sialylation of recombinant interferon-γ, *Cytotechnology* **22**, 197–203 (1996).
38 Joziasse DH, Schiphorst WECM, Van den Eijnden DH, Van Kuik JA, Van Halbeek H, Vliegenthart JFG, Branch specificity of bovine colostrum CMP-sialic acid: Galβ1 → 4GlcNAc-R α2 → 6-sialyltransferase. Sialylation of bi-, tri-, and tetraantennary oligosaccharides and glycopeptides of the *N*-acetyllactosamine type, *J Biol Chem* **262**, 2025–33 (1987).
39 Nemansky M, Schiphorst WECM, Van den Eijnden DH, Branching and elongation with lactosaminoglycan chains of N-linked oligosaccharides result in a shift toward termination with α2 → 3-linked rather than with α2 → 6-linked sialic acid residues, *FEBS Lett* **363**, 280–4 (1995).
40 Hummel M, Hedrich HC, Hasilik A, Elongation of *N*-acetyllactosamine repeats in diantennary oligosaccharides, *Eur J Biochem* **245**, 428–33 (1997).
41 Koma M, Miyagawa S, Honke K, Ikeda Y, Koyota S, Miyoshi S, Matsuda H, Tsuji S, Shirakura R, Taniguchi N, Reduction of the major xenoantigen on glycosphingolipids of swine endothelial cells by various glycosyltransferases, *Glycobiology* **10**, 745–51 (2000).
42 Sandrin M, Fodor WL, Mouhtouris E, Osman N, Cohney S, Rollins SA, Guilmette ER, Setter E, Squinto S, McKenzie IFC, Enzymatic remodelling of the carbohydrate surface of a xenogenic cell substantially reduces human antibody binding and complement-mediated cytosis, *Nature Med* **1**, 1261–7 (1995).

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