Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins

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Received 15 March 1999; accepted 4 August 1999

We have engineered two Chinese hamster ovary cell lines secreting different recombinant glycoproteins to express high levels of human β1,4-galactosyltransferase (GT, E.C. 2.4.1.38) and/or α2,3-sialyltransferase (ST, E.C. 2.4.99.6). N-linked oligosaccharide structures synthesized by cells overexpressing the glycosyltransferases showed greater homogeneity compared with control cell lines. When GT was overexpressed, oligosaccharides terminating with GlcNAc were significantly reduced compared with controls, whereas overexpression of ST resulted in sialylation of ≥90% of available branches. As expected, GT overexpression resulted in reduction of oligosaccharides terminating with GlcNAc, whereas overexpression of ST resulted in sialylation of ≥90% of available branches. The more highly sialylated glycoproteins had a significantly longer mean residence time in a rabbit model of pharmacokinetics. These experiments demonstrate the feasibility of genetically engineering cell lines to produce therapeutics with desired glycosylation patterns.

Keywords: glycosylation, glycosyltransferase overexpression, recombinant protein expression, Chinese hamster ovary cells

Recombinant glycoproteins produced by mammalian cell lines are currently being developed as therapeutics for a spectrum of diseases. Chinese hamster ovary (CHO) cells are widely used for this purpose. Data collected on glycoforms produced by CHO cells indicate that the N-linked carbohydrate structures they synthesize exhibit many features characteristic of glycans naturally occurring on human proteins[1]. For example, bi-, tri-, and tetra-antennary structures are produced with complex-type branches consisting predominantly of the disaccharide Galβ1,4 GlcNAc capped by a terminal sialic acid residue.

Incomplete synthesis of these structures often imparts a range of structural heterogeneity, which can present special problems for manufacture of recombinant glycoproteins. For molecules with pharmacokinetic properties that are strongly dependent on the structure of their complex N-linked carbohydrates, insufficient or inconsistent sialylation and galactosylation can result in variable clearance of these proteins through the asialoglycoprotein or mannose/GlcNAc receptor-mediated pathways[2,3], potentially posing a significant problem for adequate reproducible dosing of the drug. In addition, as completion of the terminal residues on the N-linked structures of recombinant glycoproteins is often affected in unpredictable ways by the cell culture environment[1,4], the potential for variations in the cell culture process to introduce structural heterogeneity to N-linked carbohydrates is a particular challenge for maintenance of product consistency.

A mammalian host expression cell line with properties that consistently allow maximal sialylation of the majority of N-linked carbohydrate structures would be a considerable advantage for the manufacture of glycoprotein therapeutics. Therefore, we explored whether reengineering CHO production cell lines to overexpress both the β1,4-galactosyltransferase (GT) and α2,3-sialyltransferase (ST) activities that catalyze the two terminal steps in N-linked oligosaccharide biosynthesis would result in synthesis of glycoprotein products with a greater proportion of fully sialylated N-glycans.

Established CHO cell lines producing either a dimeric, tissue necrosis factor receptor–IgG1 fusion protein (TNFR-IgG)[5] or a single-chain glycoprotein, T103N, N117Q, KHRR(296–299)-AAAA–tissue plasminogen activator (TNK-tPA)[6], were reengineered to overexpress ST and/or GT. The TNFR–IgG1 fusion protein contains a complex biantennary oligosaccharide in the IgG1 heavy chain region of each monomer in addition to three complex N-linked oligosaccharide structures in the binding domain. TNK-tPA, a variant of wild-type tPA that exhibits decreased plasma clearance characteristics[6], contains three complex N-linked oligosaccharide side chains. Under standard fed-batch conditions, previous characterization has shown that TNK-tPA exhibits variable degrees of undersialylation (D. Combs and T. Etcheverry, personal communication). In contrast, carbohydrates on the binding domain of TNFR-IgG exhibit undergalactosylation as well as undersialylation, whereas those in the IgG domain are predominantly unsialylated, but variably galactosylated.

In this study, we observe that more complete processing of the terminal structures of TNK-tPA and TNFR-IgG oligosaccharides slows clearance properties, imparting longer mean residence times in a rabbit model of pharmacokinetic studies.

Results

High-level expression of ST and GT in TNFR-IgG and TNK-tPA cell lines was verified by mRNA analysis (Fig. 1). Control (parental) and reengineered cell lines were cultured under identical conditions in 3L bioreactors ($N = 2$ per cell line per experiment) using standard pro-

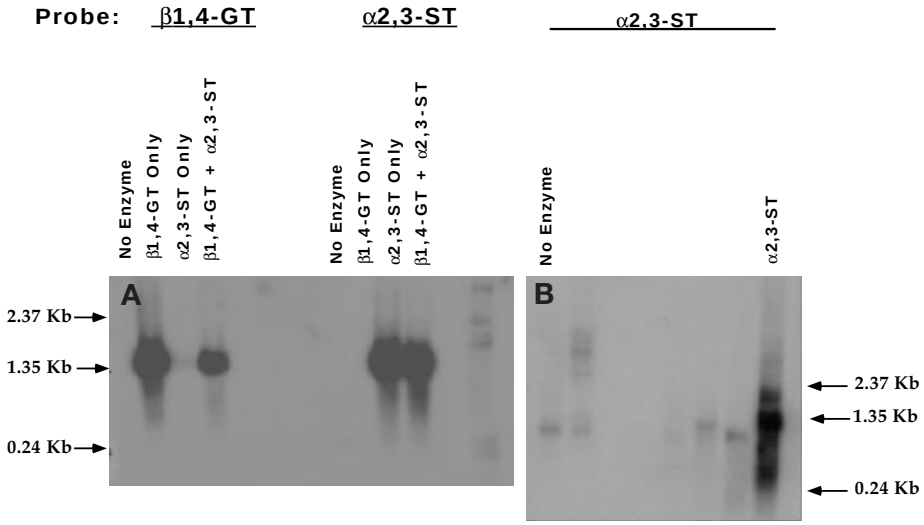

Figure 1. Northern blot analysis of GT and ST overexpression in cell lines secreting (A) TNFR-IgG and (B). TNK-tPA Five micrograms of total RNA extracted from control cells (no enzyme) or cells overexpressing GT only, ST only, or GT and ST were electrophoresed on 1% denaturing agarose gels, transferred to nylon membranes, and hybridized to ST or GT cDNA probes. Samples in unmarked lanes were from unrelated experiments.

duction conditions for each protein. Overexpression of GT and ST alone or in combination did not remarkably alter the growth or productivity of either cell line (data not shown). Cultures were terminated after eight or nine days, and the products contained in the conditioned cell culture fluids were purified by affinity chromatography and evaluated for composition by reversed-phase HPLC quantitation of *o*-phenylenediamine-2HCl (OPD) derivatized sialic acids as well as for structure using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

TNK-tPA cells overexpressing ST. To address whether overexpression of ST could alter the sialic acid content of the N-linked oligosaccharides on recombinant proteins, cells expressing TNK-tPA were transfected with ST. The negative-ion mode spectra obtained from MALDI-TOF analysis of purified TNK-tPA N-glycans from control cultures are shown in Figure 2A. Only charged, sialic acid-containing oligosaccharides were detected in the negative-ion mode. A heterogeneous mixture of structures was evident. Fully sialylated bi-, tri-, and tetra-antennary structures were present (designated as 2122, 3133, and 4144) along with structures missing sialic acid on one or more branches. Branches containing terminal sialic acid, galactose, or GlcNAc represented 77%, 21%, and 2%, respectively, of total antennae observed in the control negative-ion spectra.

In contrast, when TNK-tPA was expressed concurrently with high levels of ST, TNK-tPA contained predominantly fully sialylated forms, with a minimal amount of 2121 structures remaining (Fig. 2B). Greater than 90% of available branches were capped with sialic acid, whereas branches presumed to contain terminal galactose were decreased to <6%. These data were confirmed by quantitation of OPD derivatized sialic acids (data not shown).

The positive-ion spectra of TNK-tPA glycans purified from control cultures are shown in Figure 2C. Only neutral oligosaccharides were detected in the positive-ion mode, whereas in material from the control TNK-tPA cell line, biantennary structures with two galactoses (2120) predominate. Upon overexpression of ST, the 2120 structure was dramatically reduced (Fig. 2D), presumably because these structures are sialylated and now appear in the negative mode as 2121 or 2122 structures.

Effects on TNK-tPA clearance in vivo. The pharmacokinetics of TNK-tPA produced by control and ST-overexpressing cells were compared after intravenous bolus injection of 0.3 mg/kg into the ear vein of rabbits. As expected, more fully sialylated TNK-tPA was cleared more slowly than material expressed by control cell lines (2.0 ± 0.3 ml/min/kg for control versus 1.42 ± 0.3 ml/min/kg for ST, $P < 0.001$). This resulted in an approximately 30% increase in

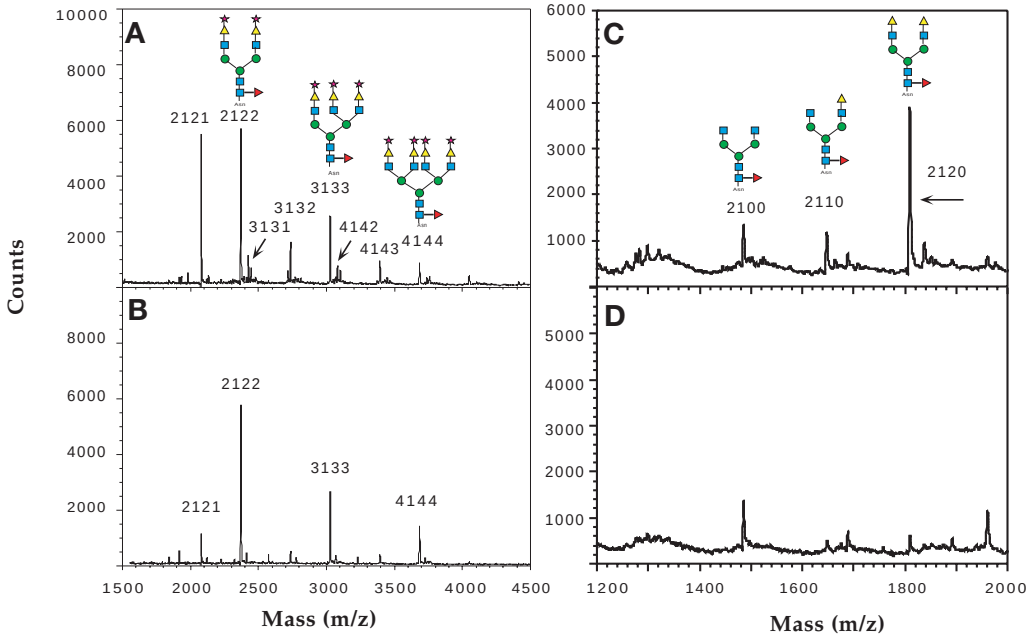

Figure 2. MALDI-TOF MS in (A and B) negative-ion mode and (C and D) positive-ion mode of oligosaccharides on TNK-tPA produced by (A and C) control cells and (B and D) cells reengineered to overexpress ST alone. Individual oligosaccharides are identified by their characteristic mass (m/z) and are designated using a four-digit code that specifies the antennarity, number of fucose residues, number of galactose residues, and the number of sialic acids present on a complex N-glycan containing a trimannose core structure. For example, a 2122 structure has a characteristic mass of 2369.2 m/z and has 2 antennae, 1 fucose, 2 galactose, and 2 sialic acid residues.

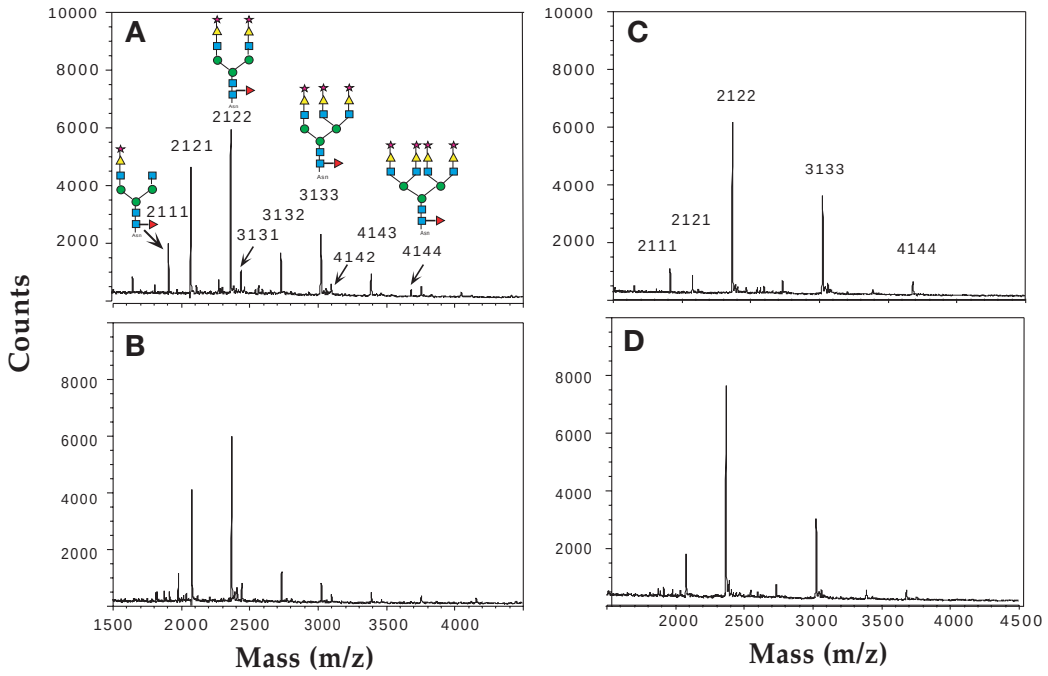

Figure 3. MALDI-TOF MS in negative-ion mode of oligosaccharides on TNFR-IgG produced by (A) control cells and cells reengineered to overexpress (B) GT alone (C) ST alone, and (D) both enzymes together (GT/ST). Individual oligosaccharides are identified as described in the legend to Figure 2.

drug exposure as measured by area under the curve (AUC) for this material (153 ± 21 μg•min/ml for control versus 217 ± 38 μg·min/ml for ST, $P < 0.001$). These data are consistent with decreased recognition of more highly sialylated structures by the asialoglycoprotein receptor. Furthermore, the data demonstrate that increases in sialic acid content, such as those realized for TNK-tPA in these experiments, can have significant impact on the pharmacokinetics of glycoprotein therapeutics.

TNFR-IgG cells overexpressing GT and/or ST. To confirm that overexpression of ST can alter the structure of N-linked glycans on other recombinant proteins and to determine whether coexpression of GT is beneficial in promoting increased sialylation and homogeneity of undergalactosylated as well as undersialylated oligosaccharide structures, a second glycoprotein, TNFR-IgG, was studied. As with TNK-tPA, a heterogeneous mixture was evident in the negative-ion spectra of TNFR-IgG N-glycans produced in control cultures (Fig. 3A). Fully sialylated bi-, tri-, and tetra-antennary structures were present along with structures missing sialic acid on one or more branches and a single structure missing both terminal galactose and sialic acid on one branch (2111). Terminal sialic acid, galactose, or GlcNAc was present on 76%, 20%, and 4%, respectively, of all the branches.

The distribution of oligosaccharide structures present on TNFR-IgG produced concurrently with overexpression of GT was similar, in the negative-ion mode, to that seen in control cultures except that antennae terminating with GlcNAc decreased from 4% to <1% (Fig. 3B). This result was expected because most of the heterogeneity observed in the negative-ion mode results from

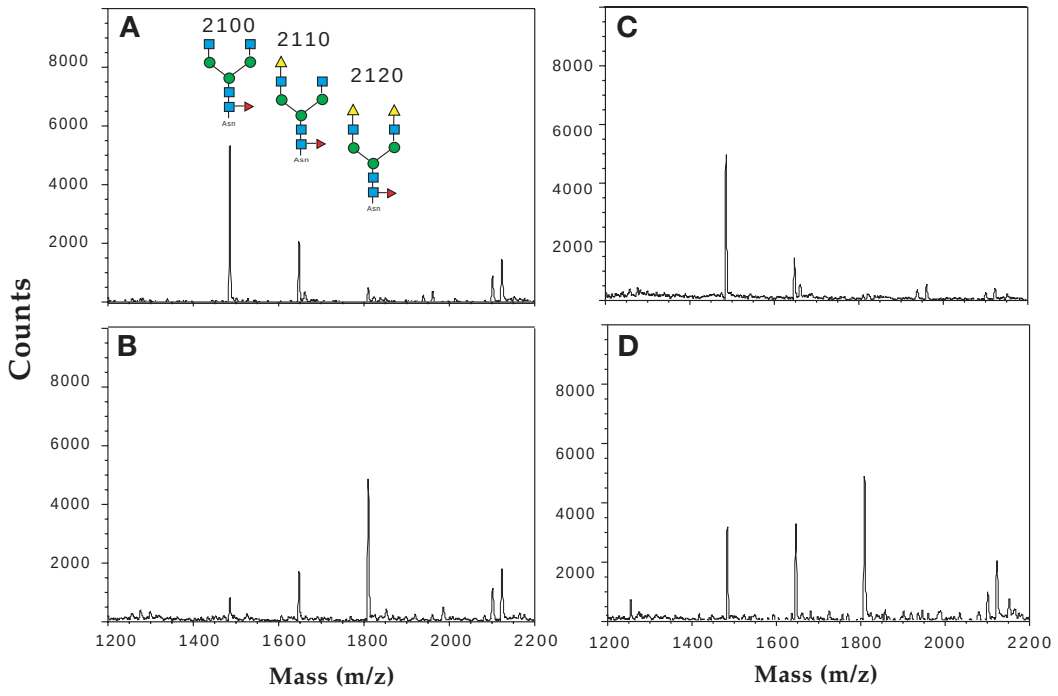

Figure 4. MALDI-TOF MS in positive-ion mode of oligosaccharides on TNFR-IgG produced by (A) control cells and cells reengineered to overexpress (B) GT alone (C) ST alone, and (D) both enzymes together (GT/ST). Individual oligosaccharides are identified as described in the legend to Figure 2.

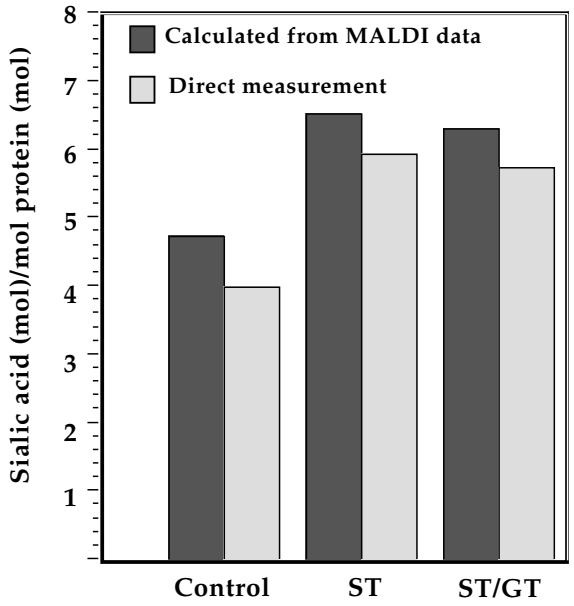

Figure 5. Sialic acid content of TNFR-IgG measured by reversed phase HPLC quantitation of OPD-derivatized sialic acids compared with the calculated content based on the antennarity and relative abundance of structures identified by MALDI-TOF MS (negative-ion spectra).

undersialylation. Conversely, when TNFR-IgG was produced by cells overexpressing ST, the heterogeneity of acidic oligosaccharides was significantly decreased compared with controls (Fig. 3A versus 3C); predominantly fully sialylated structures were observed, with only minor amounts of undersialylated material remaining (2121 and 2111). Terminal sialic acid was present on 92% of all antennae identified in the negative-ion mode. Moreover, when both glycosyltransferases were coexpressed, a decrease in glycan heterogeneity similar to that seen with ST alone was again observed along with a decrease in the presence of 2111-type structures (Fig. 3D). Again, branches terminating with sialic acid represented 90% of total oligosaccharides present in the negative-ion spectra whereas structures terminating with exposed GlcNAc were decreased to ≤1%.

The positive-ion spectra of TNFR-IgG glycans purified from control cultures are shown in Figure 4A. Only neutral oligosaccharides were detected in the positive-ion mode, and biantennary structures represent >90% of all glycans detected. These structures terminate with galactose on one or two branches (2110 or 2120) or with GlcNAc alone (2100). Figure 4A shows that the majority (65%) of oligosaccharides found in the positive mode for TNFR-IgG from control cultures are nongalactosylated, although lesser amounts of 2110 (28%) and 2120 (7%) structures can be seen. Previous characterization of TNFR-IgG (A. Jones, unpublished data) indicates that approximately 60% of glycans observed in the positive-ion mode represent structures contained in the IgG domain of the molecule, which are not readily sialylated.

Figure 4B shows that the uncharged oligosaccharides present on TNFR-IgG coexpressed with GT were dramatically shifted toward more highly galactosylated 2120 forms (68% 2120 [GT] versus 7% 2120 [Control]). Overexpression of ST alone resulted in only a modest decrease in the relative abundance of 2110 and 2120 structures and an enrichment of 2100 structures compared with control material (Fig. 4A versus C).

Similarly to results observed with overexpression of GT alone, neutral oligosaccharides containing one or two galactose residues were significantly more abundant in the positive-ion spectra of TNFR-IgG oligosaccharides produced by GT/ST cultures than in control cultures (Fig. 4A versus D). However, as in the ST-only case, there was again a relative enrichment in the percentage of 2100 structures when GT and ST were expressed in combination compared with the GT-only case (Fig. 4B versus D). Although it is not possible to reliably quantitate movement of glycoforms between the positive- and negative-ion modes, it is likely that enrichment of 2100 glycoforms in the presence of ST alone is the result of sialylation of a portion of 2110 and 2120 structures, removing them from the positive-mode spectra. As an increased number of galactosylated structures are available for sialylation when GT is coexpressed, the magnitude of this enrichment effect may be greater in the combination cases than when ST is overexpressed alone. Alternatively, the increased ratio of 2100 structures relative to 2110 and 2120 forms in GT/ST cultures—compared with controls or GT-only cultures—could stem from differences in the relative expression of GT in the cases. This could be because of either a decreased gene copy number or use of a mixed population of transfected cells for these experiments.

Results of the MALDI-TOF analysis were consistent with reversed-phase HPLC quantitation of OPD-derivatized sialic acids released from TNFR-IgG purified from each case. In general, concurrent overexpression of ST along with product resulted in a 30% increase in the molar content of sialic acid per mole of TNFR-IgG when measured directly (Fig. 5). These increases agreed well with the calculated sialic acid content predicted by the negative-mode MALDI-TOF MS data after accounting for the relative abundance of bi-, tri, and tetra-antennary structures.

Discussion

The N-linked oligosaccharides of glycoproteins contribute to protein solubility, pharmacokinetics, and in certain instances, biological activity[1,3,7–9]. For example, it is well documented that the total number of exposed galactose residues, as well as the antennarity of the structures on which they reside, plays a central role in clearance of sialylated glycoproteins in vivo[10]. In addition, the degree of galactosylation of oligosaccharides present in the Fc region of antibodies has been hypothesized to play a role in antibody clearance as well as effector function[11]. The ability to manufacture a recombinant glycoprotein with structurally consistent carbohydrates would be advantageous because it would allow maximal control over lot-to-lot analytical and biological variability[12]. Recombinant glycoproteins produced in mammalian cells such as CHO exhibit marked heterogeneity in the structure of their complex N-linked oligosaccharide side chains. Mixtures of bi-, tri-, and tetra-antennary structures are often present, which are either fully sialylated or lack sialic acid and/or galactose on one or more branches[1,3,12]. Further, the relative proportions of the various structures produced is known to be influenced either directly or indirectly by the cell culture environment, process conditions, the growth rate, and/or specific productivity of the cells[1,4,13]. The underlying cause of this heterogeneity has been hypothesized to be related to alterations of both synthetic and degradative mechanisms[1,14].

In the current studies, we have examined whether overexpression of critical glycosyltransferase enzymes involved in the addition of galactose and sialic acids to the termini of complex oligosaccharides could significantly increase the coverage of terminal branches with galactose and sialic acid and potentially provide a degree of "process robustness" to uncouple oligosaccharide quality from minor changes in cell culture processes. Results of experiments performed with two different proteins indicate that high-level expression of sialyltransferase can be highly effective in promoting consistent production of nearly fully sialylated glycoproteins. Similarly, we have shown that overexpression of galactosyltransferase is required for both maximal sialylation of all structures (providing the penultimate galactose), as well as for a consistent galactosylation pattern in nonsialylated structures, such as those

characteristic of the Fc region of antibodies and IgG domain-containing fusion proteins. Pharmacokinetic data confirm that increases in sialic acid such as those achieved in the present study can dramatically alter the clearance properties of recombinant glycoprotein therapeutics.

In our studies, the predominant acidic oligosaccharide structures (≥90%) produced by reengineered cells were fully sialylated bi-, tri-, and tetra-antennary oligosaccharides. The only quantitatively significant undersialylated structure remaining on the product produced by reengineered cells was a presumably biantennary structure missing sialic acid on one branch (2121). We have confirmed that β-galactosidase treatment of materials produced by both control and transfected cell lines removes these oligosaccharides from the MALDI-TOF spectrum (approximately 5% of 2121 remains in spectra from control and ST transfected cells; approximately 10% remains in the spectra from the GT/ST cell line), indicating that the majority of these structures do contain terminal galactose and should theoretically be sialylatable. The minimal amounts of the 2121 species that remain in TNFR-IgG and TNK-tPA, in spite of sialyltransferase overexpression, may be explained in several ways. First, postsecretory degradation of sialic acids by extracellular sialidase enzymes[14] may remove sialic acid from a portion of these structures. Alternatively, these structures may not be accessible to the ST because of either steric hindrance or a previously unappreciated branch specificity of the ST enzyme. Finally, as these cell lines are pools of transfected colonies, we cannot rule out that a proportion of the cells produce GT but not ST. These issues are the subject of ongoing studies.

The sialyltransferase selected for these studies adds sialic acid to the galactose termini of oligosaccharides in an α2,3 linkage. An enzyme with similar activity is known to be expressed naturally in CHO cells. A sialyltransferase that transfers sialic acid onto R-galactose using an α2,6 linkage is also expressed normally by many tissues of humans and other species[15,16], but not by CHO cells[17]. Thus, the example presented here represents augmentation of structures that are currently present on CHO cell–derived therapeutics.

In the present study, we have defined an improvement in glycoprotein quality as a decrease in heterogeneity resulting from an increase in the molar content of galactose and sialic acid. However, it is unclear at present what the "best" carbohydrate for each glycoprotein might be. An additional step in engineering recombinant glycoproteins may involve humanization of CHO oligosaccharides by coexpression of ST and/or the GlcNAc transferase III enzyme, which is responsible for the addition of a bisecting terminal GlcNAc residue on oligosaccharides derived from humans and other species. Other investigators have attempted to overexpress ST in production host cell lines (CHO and baby hamster kidney 21) in an effort to produce more human oligosaccharide structures[17–20]. This enzyme has been shown to compete with the endogenous ST for the donor sugar cytidine monophosphate (CMP)-sialic acid[17]. While these studies have been successful in demonstrating expression of the enzyme, as well as the addition of sialic acid on secreted[18–20] and cell surface proteins[17] in an α2,6 linkage, increases in the total sialic acid content of recombinant proteins produced by the cell have not been consistently demonstrated.

It is currently unknown whether the presence of α2,6- as well as α2,3-linked sialic acids have any significance in terms of the biological or pharmacodynamic properties of glycoproteins: These issues are being addressed in separate studies. However, our data confirm that overexpression of glycosyltransferases can significantly impact the quality of oligosaccharides on recombinant glycoproteins and suggest that it will be possible to use host cells with specifically tailored glycosynthetic profiles to predetermine the composition of the N-linked oligosaccharides produced during large-scale mammalian cell culture.

Experimental protocol

Construction of expression vectors for GT/ST overexpression. PCR products containing full-length coding sequences for the human ST (1,130 bp fragment, upper primer 5′-ATGGGACTCTTGGTATTTGT-3′, lower primer 5′-ATCTAAGCAGTGGCATCTGA-3′) and GT genes (1,390 bp fragment, upper primer 5′-CTTCTTAAAGCGGCGGCGGGAA-3′, lower primer 5′-TCACATGCCGAGCCAAGTTGGG-3′) were isolated based on published sequences[21,22] from a human placenta λ library. The sequence of the PCR products was confirmed by dideoxy sequencing according to standard methods.

Construction of unique expression vectors. The vectors used for overexpression were modified from those described by Lucas and colleagues[23]. The dihydrofolate reductase (DHFR) selectable marker present on the initial vector is replaced by the gene for puromycin resistance, and the SV40 promoter/enhancer elements are exchanged with a modified promoter (V. Chisholm, personal communication) derived from the myeloproliferative sarcoma virus[24].

Transfection and selection of GT/ST cell lines. According to manufacturer's instructions (GIBCO, BRL/Lifetechnologies, Gaithersburg, MD) we used lipofectamine to transfect 3×10^6 cells/100 mm plate from established CHO cell lines producing TNFR-IgG and TNK-tPA with plasmids containing the human GT and/or ST sequences. Briefly, 60 μl of lipofectamine were mixed with 15 μg plasmid DNA in 300 μl of a DMEM Ham's F-12–based serum-free growth media, and incubated for 30 min at room temperature before being added to the cells. Following overnight incubation at 37°C, the medium was replaced with fresh medium containing 5% diafiltered fetal bovine serum and 10 μg/ml puromycin . Colonies appearing on the plates were pooled and readapted to serum-free suspension growth. Expression of TNFR-IgG and TNK-tPA in all cell lines was maintained by addition of methotrexate to the growth media.

Cell culture and recovery of recombinant TNFR-IgG and TNK-tPA. Product quality experiments were performed in 3 L stirred-tank bioreactors (Applikon, Foster City, CA) equipped with calibrated dissolved oxygen, and pH and temperature probes. Dissolved oxygen was controlled on-line through sparging with air and/or oxygen at 60 ± 5% air saturation. The pH was maintained at 7.2 ± 0.1 and controlled on-line through the addition of CO_2 or base (Na_2CO_3). Temperature was maintained using an electric heating jacket.

Control and reengineered cell lines were cultivated under identical conditions using a standard process developed for each product. Samples were taken daily for determination of cell concentration, viability, and titer. TNFR-IgG cultures were harvested on day 9 (50–70% viability); TNK-tPA cultures were harvested after eight days (~70% viability). The harvested cell suspension was centrifuged (200 *g*, 10 min) at low speed, and the cell-free supernatant was filtered (0.2 μm). At least two independent experiments with $N = 2$ bioreactors/cell line were carried out for each protein.

The TNFR-IgG was purified by protein A affinity chromatography (ProSep Protein A, Bioprocessing, Durham, UK) using fast protein liquid chromatography. The TNK-tPA was purified by affinity chromatography using a goat anti–TNK-tPA polyclonal antibody coupled to glyceryl-controlled pore glass resin (Sigma, St. Louis, MO).

Northern blot analysis. Total RNA was extracted from parental and glycosyltransferase-transfected cell lines using the Trizol reagent as described by the manufacturer (GIBCO BRL/Lifetechnologies, Gaithersburg, MD). A 5 μg sample of of total RNA from each cell line was denatured in 50% formamide, 3% formaldehyde, 1× MAE (40 mM MOPS, 10 mM Sodium Acetate, 1 mM EDTA (pH 8.0), pH 7.0) buffer at 55–60°C for 15 min and electrophoresed in a 1% agarose gel containing 0.66 M formaldehyde. RNA was then transferred to nylon membranes (Max-S Nytran, Schleicher & Schuell, Keene, NH) using capillary electrophoresis and crosslinked to the membrane using ultraviolet irradiation (Stratalinker; Stratagene, La Jolla, CA). The membranes were hybridized to nick-translated ^{32}P-labeled cDNA probes specific for either the GT or ST transcripts using standard protocols. Hybridized probe was detected using autoradiography.

Sialic acid content determination. Sialic acids from TNFR-IgG and TNK-tPA were derivatized with OPD and analyzed on a C-18 reversed-phase HPLC column (Hewlett-Packard, Palo Alto, CA, HP 1090M) as described by Anumula[25]. The monosaccharide derivatives were detected with a fluorescence detector (HP 1046A); excitation, 230 nm; emission, 425 nm.

MALDI-TOF/MS. Oligosaccharides were released from TNFR-IgG and TNK-tPA using N-glycosidase F and analyzed by MALDI-TOF, as described[26]. Acidic oligosaccharides were analyzed in the negative-ion mode, using 2′,4′,6′-trihydroxyacetophenone (Aldrich Chemical Company,

Milwaukee, WI) as matrix. Neutral oligosaccharides were analyzed in the positive-ion mode using a 2,5-dihydroxybenzoic acid matrix. All samples were irradiated with ultraviolet light (337 nM) from an N_2 laser. The Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, MA) was used to acquire the spectra. With this method, individual oligosaccharide species can be quantitatively detected when mixtures of known quantities of acidic or neutral oligosaccharides are ionized. Spectra were acquired from at least two independent bioreactors for each case.

In vivo pharmacokinetics of TNK-tPA. The pharmacokinetics of TNK-tPA produced by control and ST-overexpressing cells were compared after intravenous bolus injection of 0.3 mg/kg into the ear vein of rabbits (N = 12–13 animals/group). Blood samples (1.5 ml) were collected at 0 (predose), 1, 2, 4, 7, 10, 15, 20, 25, 30, 40, 60, 90, 120, 180, and 240 min following dosing from the contralateral ear. Plasma was harvested and stored at -70°C until assay for TNK-tPA by ELISA. A biexponential equation was fit to the plasma concentration-time data from each individual animal, using a nonlinear least squares regression program (PCNONLIN and NONLIN84)[27]. The disposition of material from control and ST-overexpressing cell lines was characterized by calculating the plasma clearance and exposure (AUC) for each animal. Data were analyzed by one-way analysis of variance with comparison of individual means, using Fischer's Modified Least Significant Difference test. Statistical significance was defined as $P < 0.05$.

Acknowledgments

The authors would like to acknowledge and thank Mary Sliwkowski, Thomas Warner, and Thomas Ryll of the Analytical Chemistry and Cell Culture & Fermentation Research and Development Departments, Genentech, Inc., for their insightful discussions and helpful comments in the planning of these studies and the preparation of this manuscript.

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