Glycoengineering of Therapeutic Glycoproteins: In Vitro Galactosylation and Sialylation of Glycoproteins with Terminal *N*-Acetylglucosamine and Galactose Residues

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ABSTRACT: Therapeutic glycoproteins produced in different host cells by recombinant DNA technology often contain terminal GlcNAc and Gal residues. Such glycoproteins clear rapidly from the serum as a consequence of binding to the mannose receptor and/or the asialoglycoprotein receptor in the liver. To increase the serum half-life of these glycoproteins, we carried out in vitro glycosylation experiments using TNFR−IgG, an immunoadhesin molecule, as a model therapeutic glycoprotein. TNFR−IgG is a disulfide-linked dimer of a polypeptide composed of the extracellular portion of the human type 1 (p55) tumor necrosis factor receptor (TNFR) fused to the hinge and Fc regions of the human IgG_1 heavy chain. This bivalent antibody-like molecule contains four N-glycosylation sites per polypeptide, three in the receptor portion and one in the Fc. The heterogeneous N-linked oligosaccharides of TNFR−IgG contain sialic acid (Sia), Gal, and GlcNAc as terminal sugar residues. To increase the level of terminal sialylation, we regalactosylated and/or resialylated TNFR−IgG using β-1,4-galactosyltransferase (β1,4GT) and/or α-2,3-sialyltransferase (α2,3ST). Treatment of TNFR−IgG with β1,4GT and UDP-Gal, in the presence of $MnCl_2$, followed by MALDI-TOF-MS analysis of PNGase F-released *N*-glycans showed that the number of oligosaccharides with terminal GlcNAc residues was significantly decreased with a concomitant increase in the number of terminal Gal residues. Similar treatment of TNFR−IgG with α2,3ST and CMP-sialic acid (CMP-Sia), in the presence of $MnCl_2$, produced a molecule with an ∼11% increase in the level of terminal sialylation but still contained oligosaccharides with terminal GlcNAc residues. When TNFR−IgG was treated with a combination of β1,4GT and α2,3ST (either in a single step or in a stepwise fashion), the level of terminal sialylation was increased by ∼20−23%. These results suggest that in vitro galactosylation and sialylation of therapeutic glycoproteins with terminal GlcNAc and Gal residues can be achieved in a single step, and the results are similar to those for the stepwise reaction. This type of in vitro glycosylation is applicable to other glycoproteins containing terminal GlcNAc and Gal residues and could prove to be useful in increasing the serum half-life of therapeutic glycoproteins.

Protein glycosylation is a cotranslational and post-translational modification. The biosynthesis of oligosaccharides is a multistep process involving several enzymes, including glycosyltransferases and glycosidases (*1*−*3*). Often these enzymes compete with each other for a single substrate and/or an acceptor molecule. As a result, glycoproteins contain a microheterogeneous array of oligosaccharides (*4*). Hence, glycoprotein glycans are often truncated containing terminal Gal and GlcNAc residues along with sialic acid (Sia)[1] residues (*5*).

The covalently bound oligosaccharides play a significant role in the bioactivity and pharmacokinetics of proteins (*1*−*5*). Glycoproteins containing oligosaccharide structures with terminal Gal residues may be cleared rapidly from circulation as a consequence of binding to the asialoglycoprotein receptor which is present in the liver (*6*, *7*). An increase in the level of terminal sialylation of some therapeutic glycoproteins has been shown to increase their serum half-life (*6*).

Recently, Jones et al. (manuscript in preparation) has shown that terminal GlcNAc residues are involved in the rapid clearance of TNFR−IgG, an immunoadhesin molecule, presumably mediated by the mannose receptor in liver (*8*). Immunoadhesins are fusion proteins that combine the functional domain of a binding protein, usually a receptor, ligand, or cell-adhesion molecule, with immunoglobulin constant domains, usually including the hinge and Fc regions (see Figure 1). Immunoadhesins have many applications as research tools, for example, in studies of receptor−ligand interaction (*9*). The "IgG" part of an immunoadhesin may confer effector functions and increase the serum half-life of

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[1] Abbreviations: IgG, immunoglobulin G; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; β1,4GT, β-1,4-galactosyltransferase; α2,3ST, α-2,3-sialyltransferase; UDP-Gal, uridine diphosphate galactose; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; Sia, sialic acid (*N*-acetylneuraminic acid); CMP-Sia, cytidine monophosphate *N*-acetylneuraminic acid; PNGase F, peptide *N*-glycosidase F; HER2-ECD, extracellular domain of human epidermal growth factor receptor 2; RP-HPLC, reversed phase high-performance liquid chromatography; CHO, Chinese hamster ovary; rIgG, recombinant IgG; MALDI-TOF-MS, matrix-assisted laser/desorption ionization time-of-flight mass spectrometry; ESI-MS, electrospray ionization mass spectrometry.

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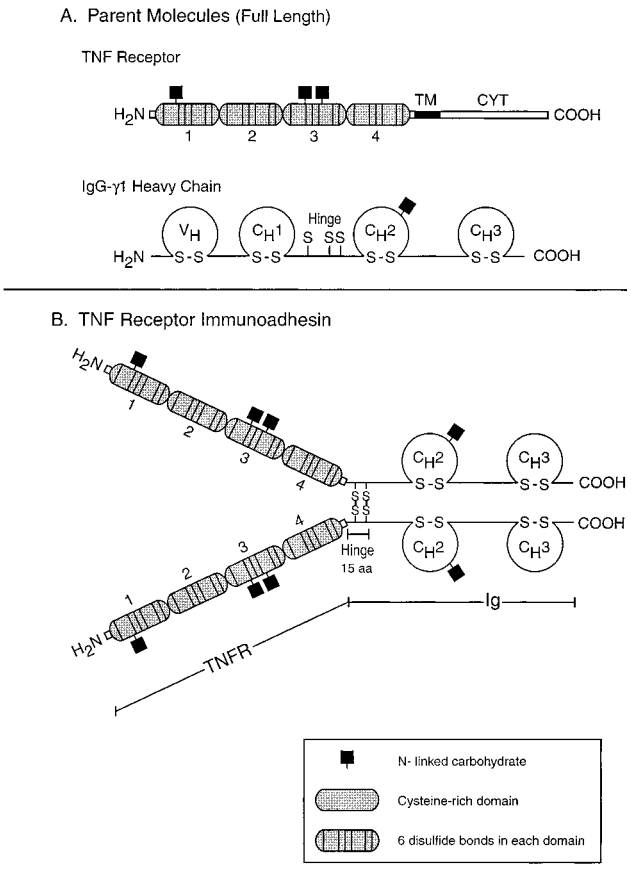


FIGURE 1: Molecular construction of TNFR−IgG showing the (A) parent molecules and (B) TNF receptor immunoadhesin. TM is the transmembrane domain, and CYT is the cytoplasmic domain of the TNFR. V_H is the variable domain, and C_H1, C_H2, and C_H3 are the constant domains of the IgG-γ1 heavy chain.

the protein, while the adhesin part determines the target specificity of the fusion protein. TNFR−IgG is composed of the extracellular portion of the type 1 (p55) tumor necrosis factor receptor (TNFR) linked to the hinge and Fc regions of the human IgG_1 heavy chain (see Figure 1). This bivalent, antibody-like molecule is a potent inhibitor of TNF, exhibiting a significantly higher affinity for TNF than the soluble form of the TNFR (*10*).

TNFR−IgG contains four N-glycosylation sites per polypeptide, three sites in the receptor region and one in the C_H2 domain of the Fc region (see Figure 1). Recombinant TNFR−IgG, expressed in Chinese hamster ovary (CHO) cells, was developed to treat patients with rheumatoid arthritis and sepsis. The N-linked oligosaccharides of TNFR−IgG are highly heterogeneous with Sia, Gal, and GlcNAc as terminal sugars, and the molecule is partially O-glycosylated in the hinge region.[2] Since TNFR−IgG molecules containing terminal GlcNAc were selectively cleared rapidly from the serum, we used in vitro glycosylation methods to increase the level of terminal sialylation to increase the serum half-life. In this paper, we describe the results of single-step in vitro galactosylation and sialylation of TNFR−IgG using a combination of β1,4GT and α2,3ST in the presence of UDP-Gal, CMP-Sia, and $MnCl_2$.

[2] Raju, T. S., Keck, R., Borge, S., and Jones, A. (1998) Affinity of *O*-Glycans for Jacalin-Agrarose in the Presence of N-Linked Oligosaccharides, Glycobiology 1998, The Annual Conference of the Society for Glycobiology, Baltimore, MD, Nov 11−14, 1998.

EXPERIMENTAL PROCEDURES

Materials. TNFR−IgG was produced and purified as described previously (*11*). Bovine β-1,4-galactosyltransferase (β1,4GT) and UDP-Gal were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant rat liver α-2,3-sialyltransferase (α2,3ST) and CMP-Sia were obtained from Calbiochem (San Diego, CA). PNGase F was obtained from New England Biolabs (Beverly, MA) or from Prozyme (San Leandro, CA). NAP-5 and protein A columns were from Pharmacia Biotech (Piscataway, NJ). All other chemicals were analytical grade.

Analytical Methods. Neutral hexoses were quantitated by the phenol−sulfuric acid assay (*12*), and the sialic acids were analyzed by RP-HPLC as described by Anumula (*13*).

In Vitro Galactosylation of TNFR−IgG. TNFR−IgG in 100 mM sodium cacodylate buffer (pH 6.4) (~10 mg in 1.0 mL of buffer) was treated with 50 milliunits of β1,4GT, 5 μmol of UDP-Gal, and 5 μmol of $MnCl_2$ at 37 °C for 24 h. Another aliquot of enzyme and UDP-Gal was added and the mixture incubated for an additional 24 h at 37 °C. The regalactosylated TNFR−IgG was purified using a HiTrap protein A column. The oligosaccharides were released by PNGase F and characterized by MALDI-TOF-MS as described below.

In Vitro Sialylation of TNFR−IgG. TNFR−IgG in 100 mM sodium cacodylate buffer (pH 6.4) (~10 mg in 1.0 mL of buffer) was treated with 50 milliunits of recombinant rat liver α2,3ST, 5 μmol of CMP-Sia, and 5 μmol of $MnCl_2$ at 37 °C for 24 h. Another aliquot of α2,3ST and CMP-Sia was added and the mixture incubated for an additional 24 h. The resialylated TNFR−IgG was purified on a HiTrap protein A column as described above and used for further analysis.

Stepwise in Vitro Galactosylation and Sialylation of TNFR−IgG. TNFR−IgG (~10 mg in 1.0 mL of buffer) was regalactosylated as described above. The regalactosylated sample was purified on a HiTrap protein A column. The sample was brought into 100 mM sodium cacodylate buffer using NAP-5 columns and resialylated as described above. The reglycosylated TNFR−IgG was purified on a HiTrap protein A column and used for further analysis.

Combined in Vitro Galactosylation and Sialylation of TNFR−IgG in a Single Step. TNFR−IgG was brought into either 100 mM sodium cacodylate or 50 mM MES buffer (pH 6.4) (~10 mg in 1.0 mL of buffer) using NAP-5 columns according to the manufacturer's suggested protocol. To this solution were added 50 milliunits each of β1,4GT and α2,3ST and 5 μmol each of UDP-Gal, CMP-Sia, and $MnCl_2$. The mixture was incubated at 37 °C. After 24 h, another aliquot of enzymes was added along with the nucleotide sugars and the mixture incubated for an additional 24 h at 37 °C. The reglycosylated TNFR−IgG was purified as described above.

MALDI-TOF-MS Analysis. Glycoprotein samples, before and after in vitro glycosylation reactions, were digested in a 96-well plate format with PNGase F in 20 mM Tris-acetate buffer (pH 8.4) for 3 h at 37 °C as described by Papac et al. (*14*). The released samples containing a mixture of acidic and neutral oligosaccharides were analyzed by MALDI-TOF-MS in the positive and negative ion modes, as described elsewhere (*14*−*16*).

Table 1: Estimation of Sialic Acid Content by the OPD Method

	sialic acid content (mol/mol of protein)[a]	% increase
control	6.4	−
GalT	6.4	0.0
SiaT	7.1	10.9
SiaT/GalT (stepwise)	7.7	20.3
combination (single step)	7.9	23.4

[a] The values are the averages of three different runs, and the observed standard deviations are ±0.1.

RESULTS

Estimation of total sialic acid content using the method described by Anumula (*13*) showed that the lot of TNFR−IgG used in this study contained ~6.4 mol of sialic acid/mol of protein (see Table 1). This amount of sialic acid present in TNFR−IgG varied from batch to batch (A. J. S. Jones et al., manuscript in preparation). N-Linked oligosaccharides of TNFR−IgG, released by PNGase F according to Papac et al. (*14*), were analyzed by MALDI-TOF-MS in the negative and positive ion modes for sialylated and neutral oligosaccharides, respectively (*14*−*16*). The mass spectra of acidic and neutral oligosaccharides are shown in panels A and B of Figure 2, respectively. The structures of oligosaccharides assigned for the observed molecular ions [$(M - H)^-$ for ions in the negative mode and $(M + Na)^+$ for ions in the positive mode] are shown in Schemes 1 and 2. The data in Figure 2A show that the acidic N-linked oligosaccharides of TNFR−IgG are complex bi-, tri-, and tetraantennary structures with one to four sialic acid residues. Some of the oligosaccharides contain terminal GlcNAc residues. For example, structures 2111, 3121, and 3122 each contain one terminal GlcNAc residue, and structure 3111 contains two terminal GlcNAc residues (see Scheme 1). Similarly, some of the neutral oligosaccharides (Figure 2B) also contain terminal GlcNAc residues. For example, structure 2110 (see Figure 2B and Scheme 2) contains one terminal GlcNAc residue; structures 2100 and 3110 contain two terminal GlcNAc residues, whereas structure 3100 carries three terminal GlcNAc residues. Since TNFR−IgG was expressed in CHO cells which normally do not express *N*-acetylglucosaminyltransferase-III, the enzyme responsible for the biosynthesis of N-linked oligosaccharides with a bisecting GlcNAc residue (*17*, *18*), the terminal GlcNAc residues observed in TNFR−IgG are probably not due to oligosaccharides containing bisecting GlcNAc residues. Structures 2100 and 2110 are mostly located in the Fc region,[3] whereas structures 3100 and 3110 are located in the receptor domain of the molecule.

Pharmacokinetic studies revealed that terminal GlcNAc residues of glycans in the receptor domain are involved in the rapid clearance of TNFR−IgG from circulation (A. J. S. Jones et al., manuscript in preparation). To minimize the content of terminal GlcNAc residues, we carried out in vitro galactosylation of TNFR−IgG with bovine β1,4GT using UDP-Gal as the donor sugar. As expected, estimation of sialic acid content after regalactosylation did not show an increase

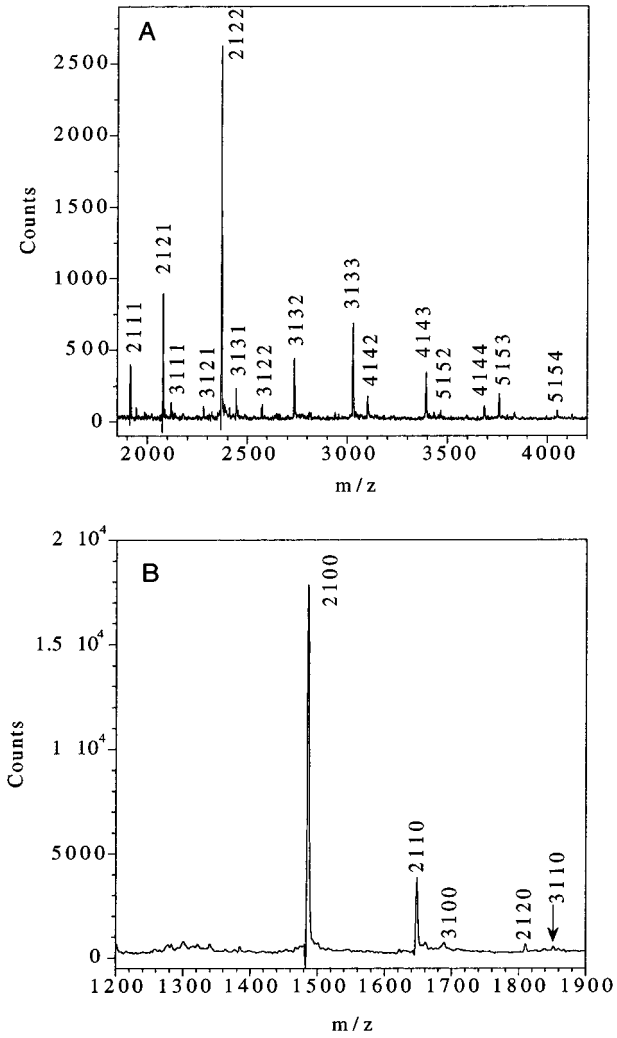


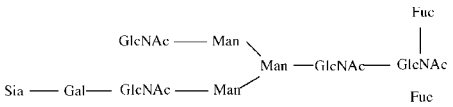
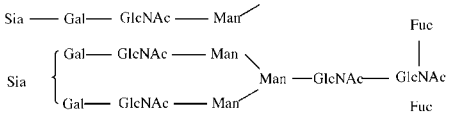
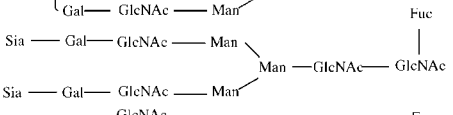
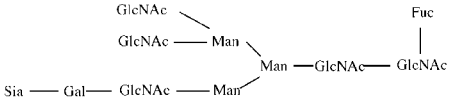
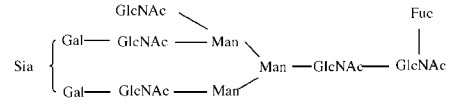
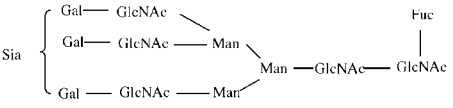
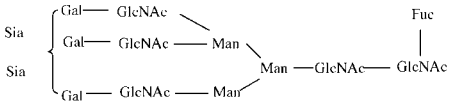
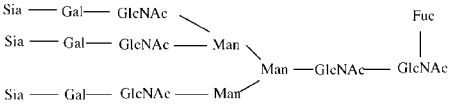
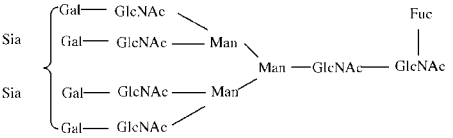
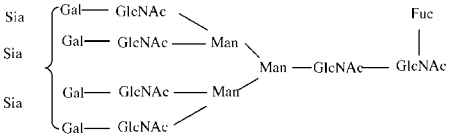
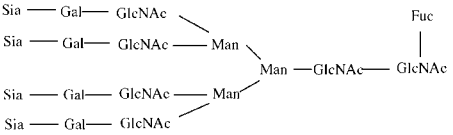
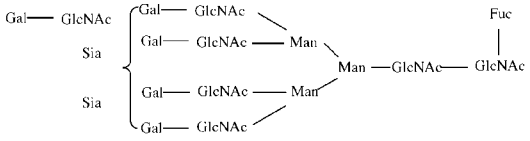
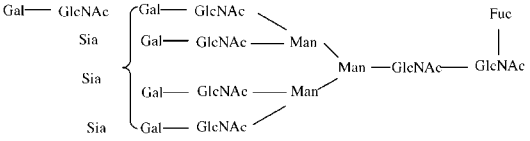
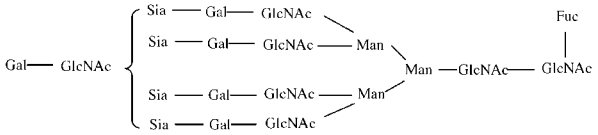
FIGURE 2: MALDI-TOF-MS of PNGase F-released N-linked oligosaccharides of TNFR−IgG. The N-linked oligosaccharides of TNFR−IgG were released and analyzed by MALDI-TOF-MS in the (A) negative ion mode using a THAP matrix and (B) positive ion mode using a DHB matrix as described in Experimental Procedures.

(see Table 1). MALDI-TOF-MS analysis of N-linked oligosaccharides released by PNGase F from the regalactosylated TNFR−IgG showed that most of the terminal GlcNAc-containing oligosaccharides were either reduced or absent after regalactosylation (see panels A and B of Figure 3). For example, the negative ion mode MALDI-TOF-MS showed that structures 2111, 3121, and 3122 were absent after regalactosylation of TNFR−IgG with β1,4GT (Figure 3A). Additional data also support this observation. For example, the negative mode MALDI-TOF-MS showed an increase in the intensity of ions corresponding to fully galactosylated structures such as 2121, 3131, and 3132 (see Figure 3A). In the positive ion mode mass spectra, the proportion of structures 2100 and 2110 was significantly decreased, while the proportion of structure 2120 was correspondingly increased. These results show that β1,4GT can also add a Gal residue to terminal GlcNAc residues present in the oligosaccharides located in the Fc region of TNFR−IgG.[4] Further,

[3] Raju, T. S., et al. (1997) Biological Significance of Glycosylation of Immunoglobins: Studies Towards the Role of Terminal Sugar Residues, Glycobiology 1997, The Annual Conference of the Society for Glycobiology, Long Beach, CA, Nov 1−4, 1997.

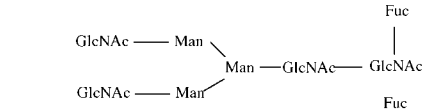
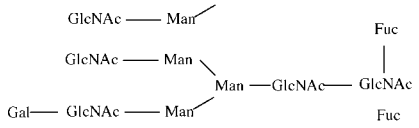
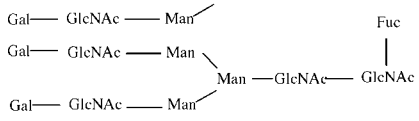
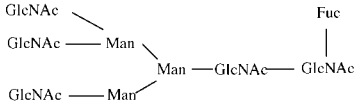
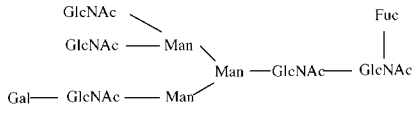
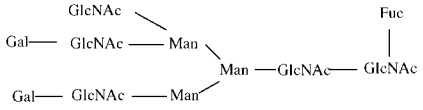
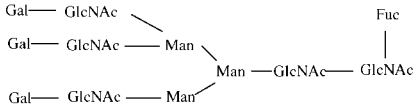
[4] Raju, T. S., et al. (1998) Biological Significance and Structural Features of Immunoglobin Glycoforms, 215th American Chemical Society National Meeting, Dallas, TX, March 29−April 2, 1998.

Scheme 1: Structure of N-Linked Oligosaccharides Found on TNFR−IgG by Negative Ion Mode MALDI-TOF-MS[a]

Structural Codes	Structure	$(M-H)^-$
2111		1915.8
2121		2077.9
2122		2369.2
3111*		2119.0
3121*		2281.1
3131*		2443.2
3132*		2734.5
3133*		3025.4
4142*		3099.8
4143*		3391.0
4144		3682.4
5152*		3465.7
5153*		3756.7
5154*		4047.7

[a] Sia, *N*-acetylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose. For oligosaccharides denoted with an asterisk, only one possible isomeric structure is shown. For the structural codes, the first digit is the number of GlcNac residues in the antennae of the N-linked oligosaccharide, the second digit is the number of Fuc residues, the third digit is the number of Gal residues, and the last digit is the number of sialic acid residues. The three Man residues and the two GlcNAc residues present in the common trimannose core region of N-linked oligosaccharides are not included in the structural code.

Scheme 2: Structure of N-Linked Oligosaccharides Found on TNFR−IgG by Positive Ion Mode MALDI-TOF-MS[a]

Structural Codes	Structure	$(M+Na)^+$
2100		1486.3
2110*		1648.5
2120		1810.6
3100*		1689.5
3110*		1851.7
3120*		2013.8
3130*		2175.9

[a] Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose. For oligosaccharides denoted with an asterisk, only one possible isomeric structure is shown. The structural codes are explained in the footnote of Scheme 1.

positive ion mode mass spectra suggest that structures 3100 and 3110 were absent, and a new structure, 3130, was observed after regalactosylation of TNFR−IgG. As expected, no significant change in the proportion of acidic oligosaccharide structures 2122, 3133, 4144, 5153, and 5154 was observed because these oligosaccharides did not contain terminal GlcNAc residues (see Scheme 1). These results suggest that the observed terminal GlcNAc residues of N-linked oligosaccharides must be caused by either undergalactosylation or clipping by galactosidases during or after the biosynthesis of TNFR−IgG because they are indeed capable of being galactosylated. Additionally, these results also confirm that terminal GlcNAc residues present in TNFR−IgG prior to regalactosylation are not due to the presence of oligosaccharides with bisecting GlcNAc residues because β-1,4-galactosyltransferase does not transfer Gal from UDP−Gal to bisecting GlcNAc residues of N-linked oligosaccharides (*19*).

From the data shown in Figure 2, it is evident that some N-linked oligosaccharides of TNFR−IgG also contain one or two terminal Gal residues in addition to terminal GlcNAc residues. For example, structure 3121 contains one terminal Gal and one terminal GlcNAc residue. Structures 3131, 4142, and 5153 contain two terminal Gal residues, whereas structures 3132 and 4143 contain one terminal Gal residue. Several previous studies showed that some asialoglycoproteins, notably those bearing oligosaccharides with three or four terminal Gal residues, can be recognized by the asialoglycoprotein receptor present in the liver and hence rapidly cleared from the serum (for a review, see ref *7*). Galactosylation of terminal GlcNAc-containing oligosaccharides could lead to an increase in the level of structures such as 3130 (Figure 3B) which are recognized by the asialoglycoprotein receptor. For this reason, we carried out in vitro sialylation of TNFR−IgG with a recombinant rat liver α2,3ST using CMP-Sia as the donor sugar. Estimation of the sialic acid content of TNFR−IgG after resialylation showed a ~10.9% increase (Table 1). The N-linked oligosaccharides of resialylated TNFR−IgG were released by PNGase F and analyzed by MALDI-TOF-MS in the positive and negative ion modes. The data shown in panels C and D of Figure 3 suggest that most of the terminal Gal-containing oligosaccharides were either absent or reduced in proportion after resialylation with α2,3ST. For example, the negative ion mode mass spectra indicated that the proportion of structures 2121 and 3132, which contained one terminal Gal residue per oligosaccharide chain, decreased with a proportional increase in the intensity of structures 2122 and 3133. Further, structures 3121, 3131, 4142, and 5152 were absent after resialylation of TNFR−IgG (see Figure 3C). Positive ion mode spectra showed that the distribution of neutral oligosaccharides remained essentially unchanged (see Figure 3D).

From Figure 3C, it is evident that even after resialylation of TNFR−IgG with α2,3ST, some N-linked oligosaccharide structures such as 2111 and 3111 are not fully sialylated. These oligosaccharides contain one and two terminal GlcNAc residues, respectively (see Scheme 1). These oligosaccharides must first be regalactosylated to obtain maximally sialylated TNFR−IgG. Hence, TNFR−IgG was first regalactosylated with β1,4-GT and UDP-Gal and purified on a protein A column. The regalactosylated TNFR−IgG was then resialylated with α2,3ST and CMP-Sia. Estimation of sialic acid content showed a ~20% increase after regalactosylation and

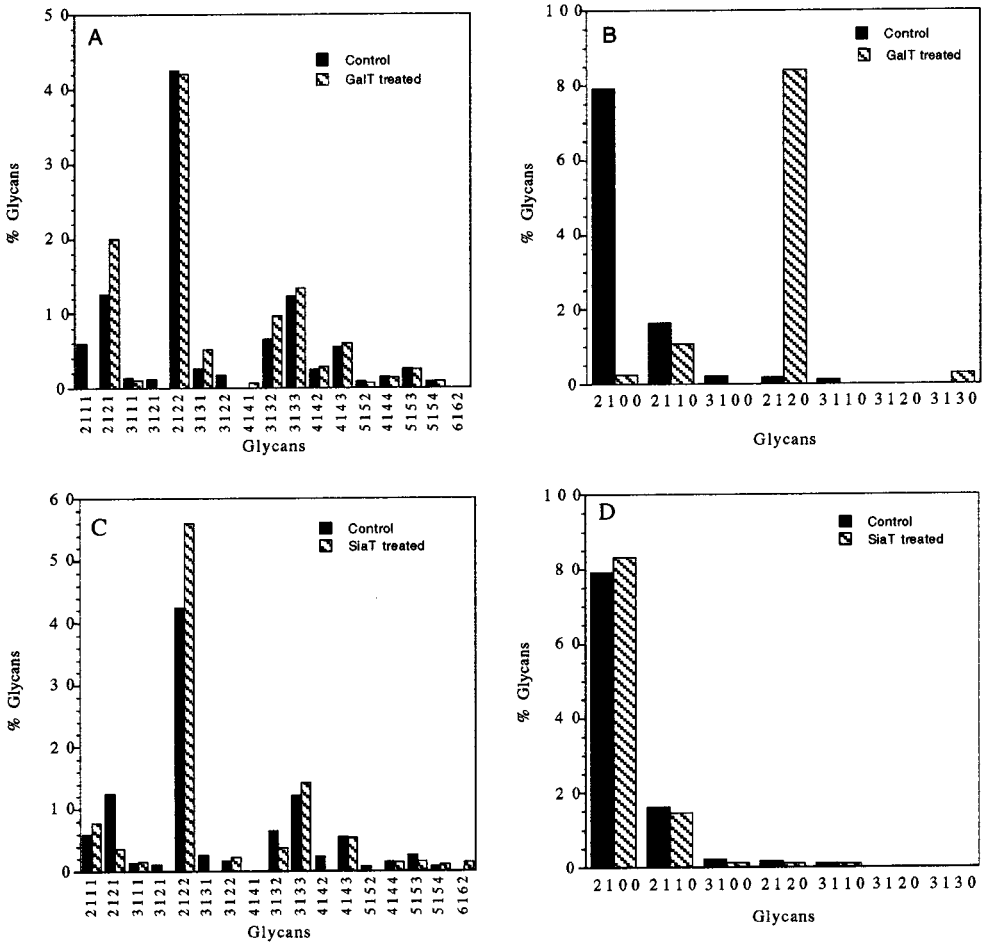


FIGURE 3: Comparison of N-linked oligosaccharides of regalactosylated (A and B) and resialylated (C and D) TNFR−IgG with control. The N-linked oligosaccharides of TNFR−IgG, before and after reglycosylation, were released by PNGase F as described in Experimental Procedures. The released oligosaccharides were analyzed by MALDI-TOF-MS in the negative ion mode for sialylated oligosaccharides (A and C) and in the positive ion mode for neutral oligosaccharides (B and D). The proportion of different oligosaccharide structures was compared.

resialylation (see Table 1). Analysis of PNGase F-released N-linked oligosaccharides of regalactosylated and then resialylated TNFR−IgG by MALDI-TOF-MS was carried out in the positive and negative ion mode. The mass spectral data are shown in panels A and B of Figure 4. In this case, the negative ion mode MALDI-TOF-MS (Figure 4A) showed that structures 3121, 3131, 3122, and 5152 were completely absent after regalactosylation and resialylation. In addition, the proportion of structures 2111, 2121, 3132, and 4142 was significantly reduced with a concomitant increase in the intensity of structures 2122, 3133, 4143, 4144, and 5154 (see Figure 4A). The N-linked oligosaccharides with structures 2122, 3133, and 4144 are bi-, tri-, and tetraantennary structures, respectively, and contained only sialic acid as the terminal sugar residue (see Scheme 1). Further, structures 4143 and 5154 might be tri- and tetra-antennary structures with one or two lactosamine repeating units. The oligosaccharides with lactosamine repeating units are also termed polylactosamine chain-containing oligosaccharides. The positive ion mode mass spectra suggested that the proportion of structures 2100 and 2110 was significantly reduced, while structures 3100 and 3110 were completely absent after regalactosylation and resialylation (see Figure 4B). The absence of structures 3100 and 3110 in the positive ion mode spectra suggested that these were converted into sialylated oligosaccharides. However, as with the galactosylation reaction alone, the 2100 and 2110 structures appear to be mainly converted to 2120 with no addition of sialic acid because they are mainly present in the Fc domain.

Although the stepwise regalactosylation and resialylation was efficient in achieving the maximum sialylation of TNFR−IgG, the procedure involves a multistep process and exposes the protein to repeated treatment with enzymes. To minimize the time of treatment and extra steps in the purification process, we treated TNFR−IgG with a combination of bovine β1,4-GT and recombinant rat liver α2,3ST in the presence of UDP-Gal and CMP-Sia as donor sugars in a single step. Total sialic acid analysis of TNFR−IgG showed an ~23% increase in sialic acid content after the combination reaction (see Table 1). MALDI-TOF-MS data of the PNGase F-released N-linked oligosaccharides of TNFR−IgG, before and after the combined regalactosylation and resialylation in a single step, were very similar to the data obtained upon stepwise regalactosylation and resialylation (see Figure 4A−D; compare panel A to C and panel B to D). The combination reaction results indicate that the regalactosylation and resialylation of N-linked oligosaccharides containing terminal GlcNAc and Gal can be achieved in a single step using a mixture of galactosyltransferase and sialyltransferase. The resulting product is equivalent to that where the enzymes are added in a stepwise fashion.

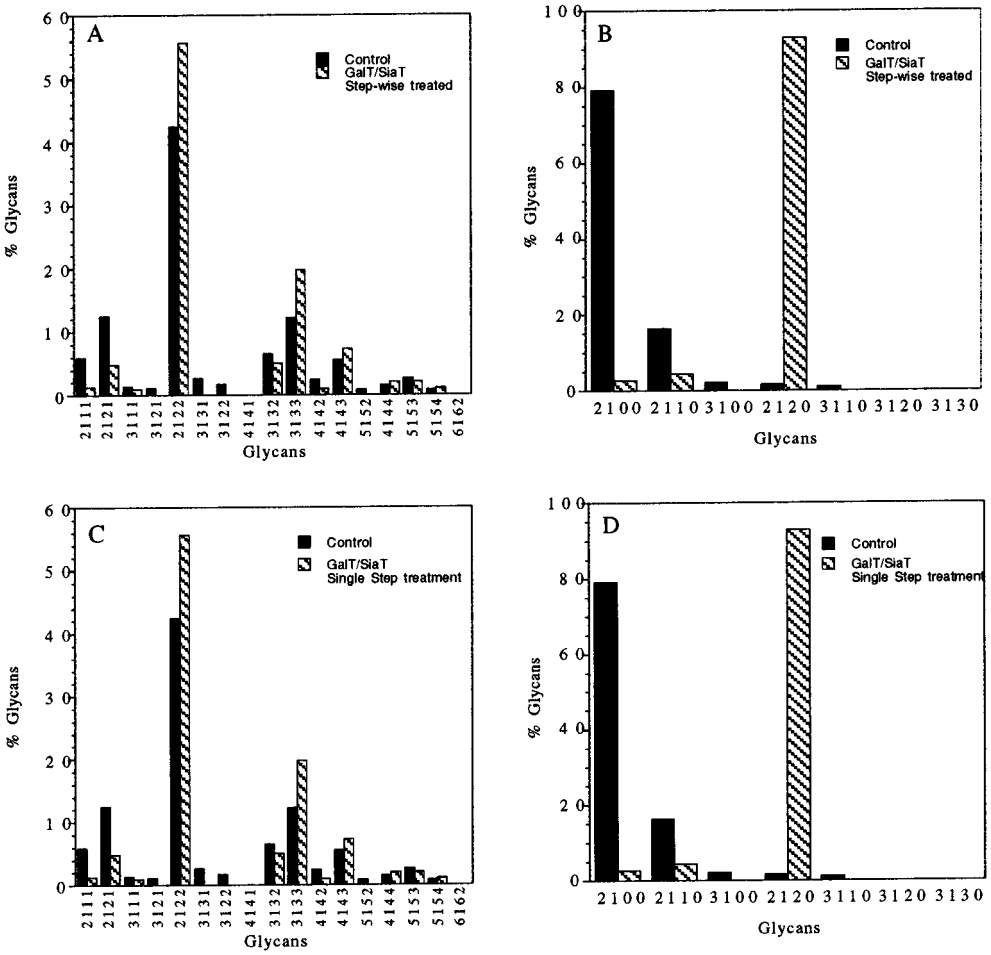


FIGURE 4: Comparison of N-linked oligosaccharides of TNFR−IgG, before and after in vitro galactosylation and sialylation. (A and B) TNFR−IgG was first galactosylated with β1,4GT in the presence of UDP-Gal and $MnCl_2$. The product was purified on a protein A column and then sialylated with α2,3ST in the presence of CMP-Sia and $MnCl_2$ as described in Experimental Procedures. (C and D) TNFR−IgG was treated with a mixture of β1,4GT and α2,3ST as described in Experimental Procedures. The N-linked oligosaccharides of both reglycosylated and control samples of TNFR−IgG were released by PNGase F and analyzed in the negative ion mode (A and C) and in the positive ion mode (B and D) via MALDI-TOF-MS, and their proportions were compared.

The combined regalactosylation and resialylation of TNFR−IgG in a single step was carried out in two different buffer systems (i.e., sodium cacodylate buffer and MES buffer), and the results of the analysis of PNGase F-released oligosaccharides by MALDI-TOF-MS are shown in Figure 5. These results suggest that the reglycosylation of TNFR−IgG in both buffer systems is efficient.

In both stepwise and combination reactions, a large proportion of structure 2120 was observed in the positive ion mode spectra which could have been converted into 2122 after regalactosylation and resialylation (see Figure 4A−D). As mentioned earlier, TNFR−IgG contains four sites for N-glycosylation, one in the CH2 domain of the Fc portion and the other three in the receptor portion. Human IgG Fc glycans are normally biantennary complex structures with or without core Fuc and bisecting GlcNAc residues. Parekh et al. (*20*) reported that only a very small amount of human IgG glycans are sialylated. Further, we recently reported the analysis of N-linked oligosaccharides derived from the serum IgGs of 13 different animal species (*16*). In that study, we found evidence for species specific variations in the N-linked oligosaccharide structures present in the Fc regions of IgGs. The results of this study suggested that the IgG glycans present in the Fc region are mainly complex biantennary structures with variations with respect to bisecting GlcNAc, core Fuc, and terminal Gal residues. The only exception was chicken IgG that contained a mixture of high-mannose and complex biantennary structures. The proportion of sialylated oligsoaccharides found in the IgGs of different animal species was relatively small (*16*). This might be due to the spatial constraints in positioning the sialylated oligosaccharides between the two-polypeptide chains in the CH2 domain of the Fc. This would explain why a large proportion of 2120 structures derived from the Fc oligosaccharides of TNFR−IgG remained nonsialylated even after being treated with galactosyltransferase and sialyltransferase.

DISCUSSION

Many glycosyltransferases are Golgi-localized enzymes which catalyze the transfer of sugar residues from nucleotide sugar donors (activated sugars) to glycoconjugates. These transferases exhibit a very high acceptor specificity, and regio- and stereoselectivity. For example, β1,4GT mediates the transfer of Gal from UDP-Gal to GlcNAc (GlcNAc can be a monosaccharide or covalently bound to other sugars) at the O-4 position of the sugar in the β-configuration. Similarly, α2,3ST mediates the transfer of sialic acid from the CMP-Sia to terminal Gal residues at the O-3 position of

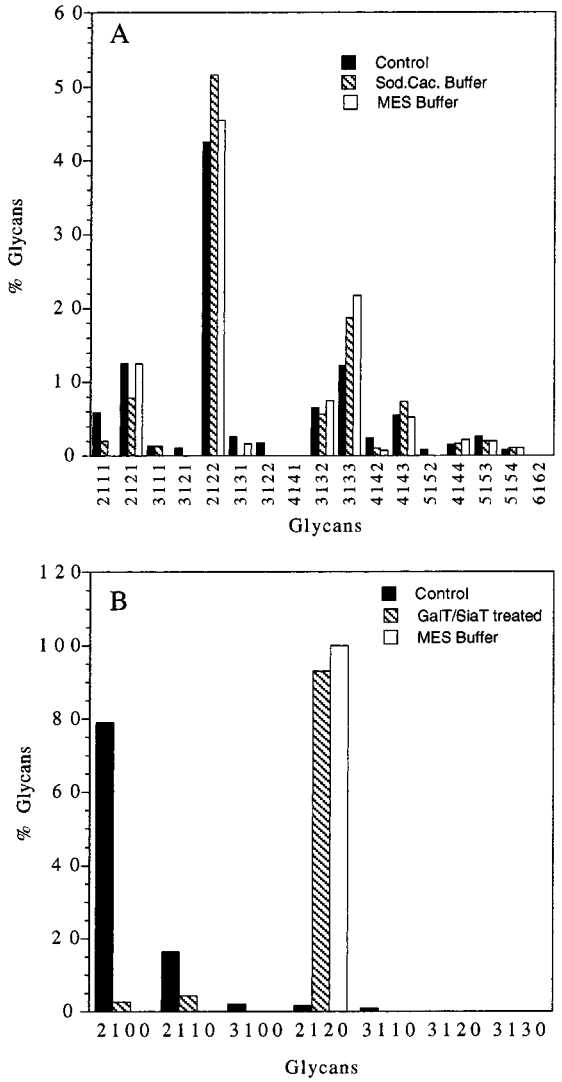


FIGURE 5: Single-step in vitro galactosylation and sialylation of TNFR−IgG in sodium cacodylate and MES buffers. In vitro galactosylation and sialylation of TNFR−IgG in sodium cacodylate buffer or in MES buffer was carried out as described in Experimental Procedures. The oligosaccharides were released and analyzed by MALDI-TOF-MS in the (A) negative ion mode and (B) positive ion mode. A comparison of the proportion of oligosaccharides is shown.

the sugar in the α-configuration, while α-1,6-fucosyltransferase transfers Fuc from GDP-Fuc to GlcNAc attached to Asn at O-6 in the α-configuration. It has been postulated that more than 200 glycosyltransferases exist in a single living cell (*3*, *21*). Each glycosyltransferase is specific for a particular linkage, a particular acceptor, and a specific anomeric configuration. More than 30 of these transferases have been cloned from prokaryotic and eukaryotic sources (*22*−*24*).

Glycosyltransferases are useful tools for in vitro synthesis of oligosaccharides and glycoconjugates. Indeed, the use of these enzymes in the pharmaceutical industry to synthesize oligosaccharides on a large scale for human therapy and to develop carbohydrate-based vaccines is very novel (*25*). A glycosyltransferase-based approach to synthesizing oligosaccharides obviates the demand for the cumbersome protection and/or deprotection and chemical glycosylation steps that are required in synthetic chemical approaches. However, the enzymatic approach requires nucleotide sugars that are expensive and difficult to synthesize. To circumvent this problem, Wong et al. (*26*, *27*) developed a recycling procedure with in situ regeneration of CMP-Sia in a one-pot reaction to synthesize sialylated oligosaccharides. Hitherto in these enzymatic reactions only one glycosyltransferase was used to add one sugar at a time to synthesize oligosaccharides. In this paper, we present evidence to show that a combination of β1,4GT and α2,3ST could be used to regalactosylate and resialylate glycoprotein glycans containing terminal GlcNAc and Gal in a single reaction step, to reduce microheterogeneity and to increase the level of terminal sialylation. The data in Figure 4 and Table 1 indicate that the combination reaction with β1,4GT and α2,3ST reduced the microheterogeneity of N-linked oligosaccharides present in TNFR−IgG. Results of the combination reaction were comparable to the results of stepwise regalactosylation and resialylation of TNFR−IgG in two different steps (see panels A and B of Figure 4). The combined reaction was carried out in two buffer systems. The data shown in Figure 5 suggest that regalactosylation and resialylation in sodium cacodylate and MES buffers are comparable. The combination reaction was used to reglycosylate other glycoproteins to increase the level of terminal sialylation. For example, regalactosylation and resialylation of HER2-ECD was carried out in a single step to increase the level of terminal sialylation (T. S. Raju et al., manuscript in preparation). Pharmacokinetic studies revealed that the reglycosylated HER2-ECD exhibited a 4−5-fold increased serum half-life compared to the control (T. S. Raju et al., unpublished data). These results also suggest that in vitro glycosylation of glycoproteins is a useful tool for studying the structure−function relationship of glycoprotein glycans.

Recently, Weikert et al. (*28*) engineered CHO cells by overexpressing human β1,4GT and α2,3ST. The N-linked oligosaccharides of recombinant glycoproteins produced in these engineered cell lines exhibited greater homogeneity compared with the control cell lines and contained increased amounts of terminal sialic acid residues. Further, the glycoproteins produced in these cell lines had a significantly longer mean residence time in a rabbit model of pharmacokinetics, confirming that increasing the sialic acid content increases the serum half-life. Our novel single-step in vitro glycosylation method also produces therapeutic glycoproteins with increased sialic acid content and decreases microheterogeneity. Further, the in vitro glycosylation of glycoproteins using multiple enzymes in a single reaction is useful and efficient in producing homogeneously glycosylated glycoproteins. The in vitro glycosylation reactions can be very easily scaled up for industrial production of therapeutic glycoproteins. Hence, we believe that the results of this study are very useful in increasing the serum half-life of therapeutic glycoproteins.

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