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Site-Specific Glycosylation of an Aglycosylated Human IgG1-Fc Antibody Protein Generates Neoglycoproteins with Enhanced Function

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Summary

A range of well-defined IgG glycoforms was prepared by employing a combination of synthetic carbohydrate chemistry and genetic engineering. The key aspect of this methodology is the coupling of thioaldoses with cysteine-containing proteins to give disulfide-linked neoglycoproteins. This technology was applied to the synthesis of a series of synthetic *N*-glycan thioaldoses which were coupled to an aglycosylated IgG1-Fc fragment, engineered to have Cys-297 in place of glycan-linked Asn (Δh-Fc N297C). Analysis of the resulting Fc neoglycoproteins by mass spectrometry and trypsin digestion showed that the saccharides were site-selectively incorporated at Cys-297 to full occupancy without affecting other Fc protein disulfides. The neoglycoproteins were tested for their ability to interact with human FcγRI by inhibiting superoxide production by γ-interferon-stimulated U937 cells. The neoglycoproteins displayed enhanced superoxide inhibition relative to aglycosylated Δh-Fc N297C, where increased glycan size correlated positively with increased inhibition.

Introduction

Protein glycosylation is an important cotranslational modification, which has been implicated as playing major roles in modulating protein folding and stability, cell-cell communication and receptor binding [1–6]. As oligosaccharide synthesis is not under direct genetic control, ensuing protein glycosylation results in the formation of a heterogeneous range of glycoforms that possess the same peptide backbone but differ in the nature and site of glycosylation. It has been suggested that variations in saccharide substitution is a means of fine-tuning bioactivity of glycoproteins [7]. However, due to difficulties of obtaining well-defined glycoforms, it has only been possible in a few cases to determine the biological roles of individual glycoforms. Furthermore, it is difficult in general to control glycoform formation in cell culture, which is a major obstacle for the development of therapeutic glycoproteins [8].

Immunoglobulin G (IgG) antibodies provide an intriguing example of functional diversity orchestrated through glycosylation [9]. The IgG molecule is a multifunctional glycoprotein that binds antigens (pathogens) specifically to form immune complexes that activate effector mechanisms resulting in the clearance and destruction of pathogens. IgG antibodies are comprised of two identical antigen binding (Fab) units, which are linked via a flexible hinge to the Fc unit (Figure 1). After antigen binding, the Fc unit can activate a range of effector responses, which include the complement cascade and cellular defense mechanisms such as superoxide production triggered by binding to Fcγ receptors. The IgG-Fc region is a homodimer comprising interchain disulfide-linked hinge regions, glycosylated C_H2 domains bearing *N*-linked oligosaccharides at asparagine 297 (Asn-297), and noncovalently paired C_H3 domains. Several elegant studies [9, 10] have shown that glycosylation of the Fc region is essential for many of its effector functions. There is evidence to suggest that the oligosaccharides exert their effects through stabilization of the protein conformation [11, 12]. In particular, X-ray crystallographic studies have shown that the oligosaccharide is sequestered within the internal space enclosed by the C_H2 domains making multiple contacts with the protein [13]. A recent X-ray crystallographic study [14] of an Fc unit bound to a truncated FcχRIII fragment revealed that the *N*-glycan of the Fc unit forms a contact with the receptor involving a potential hydrogen bond.

The oligosaccharide moiety at Asn-297 is of the complex biantennary type displaying considerable microheterogeneity. The latter feature seems to be important for Fc-function and disease, and for example, changes in glycosylation have been observed for a number of inflammatory diseases that include rheumatoid arthritis [15–17] and systemic vasculitis [18]. Patients with such diseases show a deficit in galactosylation resulting in a high proportion of IgG molecules bearing oligosaccharides terminating in *N*-acetyl glucosamine. Immune complexes formed through these glycoforms can bind mannan binding lectin, thereby activating a pseudoclassical complement cascade [16].

There is an urgent need for a method that enables the production of a wide variety of homogenous glycoforms of recombinant IgG. Such approaches would not only be of great use to determine biological roles of individual glycoforms but would also be valuable for the production of therapeutic antibodies.

We report here a novel approach for obtaining a range of well-defined glycoforms of IgG1-Fc by asymmetric disulfide conjugation between synthetic thioaldoses and a recombinant IgG-Fc protein having its natural asparagine glycosylation site replaced with a cysteine residue. The effector capabilities of the resulting Fc neoglycoproteins were evaluated by their ability to interact with hu-

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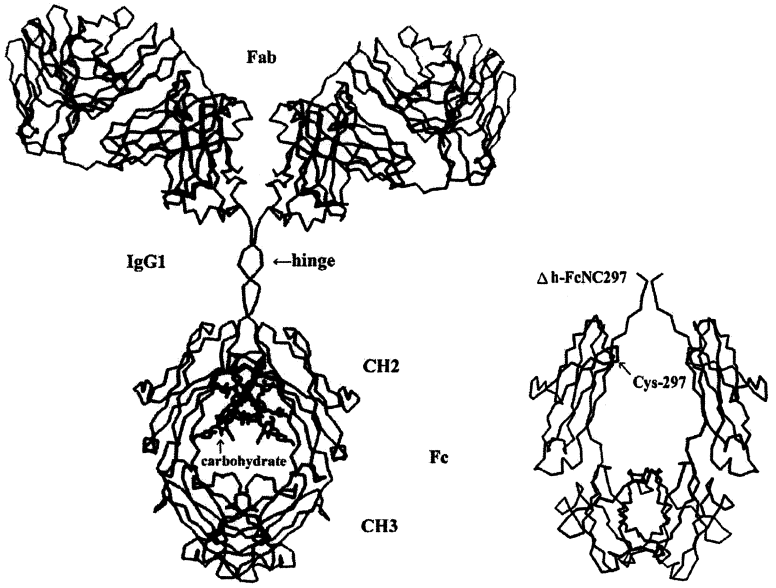

Figure 1. Schematic Representations of Human IgG1 and Truncated Hingeless Fc

The representation of the intact IgG1 structure is taken from [42]. Truncated hingeless Fc (Δh-Fc N297C) (residues 231–447 of the heavy chains) contains the C_H2 and C_H3 domains, but lacks the hinge region (residues 216–230) and the complex oligosaccharide moieties attached to residue 297. The two heavy chains are held together by noncovalent pairing of the C_H3 domains.

man Fc γ-receptor I by inhibiting a superoxide burst from γ-interferon-stimulated U937 cells.

Results and Discussion

The de novo synthesis offers a promising approach to well-defined glycoforms of glycoproteins, and several methods have been explored, each representing unique and difficult challenges [19]. The chemical synthesis of glycoproteins using standard peptide synthesis is only feasible for peptides smaller than 30 amino acid residues. State of the art native ligation techniques [20] make it possible to couple (glyco)peptide fragments to afford more complex glycopeptides [2, 21–25]. While synthetic glycopeptides and glycoproteins have proven to be indispensable probes for glycobiology, parallel efforts toward the synthesis of glycopeptide and glycoprotein mimetics are being undertaken [24]. These analogs may have improved pharmacokinetic properties and are invaluable tools for understanding carbohydrate-lectin interactions, proximal peptide-sugar conformational requirements, and the synthesis of immunostimulating glycoconjugates [4, 24, 26–28]. Most synthetic targets, however, have been rather small with respects to peptide length, representing fragments of glycoproteins several orders of magnitude larger in size.

Previously we communicated [29] a novel approach for site-selective coupling of saccharides to proteins by the condensation of a nitropyridinesulfenyl thioglycoside with a free thiol of a cysteine residue of a peptide or protein. This approach exploits the fact that cysteines in proteins are normally present as disulfides. An additional cysteine moiety with a free thiol can, however, be introduced at any point of a polypeptide chain by site-directed mutagenesis. The resulting highly nucleophilic thiol can then be reacted with a sugar bearing an electrophilic moiety to give a well-defined neoglycoprotein. In a seminal study, Flitsch and coworkers have coupled a monosaccharide bearing an iodoacetamide group to a human erythropoietin cysteine mutant [30]. Although site-selective glycosylation of the protein was achieved, the resulting thioacetamide glycan-protein linkage is longer by two atoms than the natural linkage to asparagine. With this increased linkage length it may not be possible to reproduce contact points between oligosaccharide and peptide chain which have been found to be important in IgG [31]. We have shown that a disulfide linkage between a thioaldose and cysteine can adopt a conformation similar to that of the *trans* amide linkage often found in natural *N*-linked glycoproteins, and the unusual *cis* conformation found in IgG [32]. Thus, it was envisaged that a range of functional and homogenous glycoforms of IgG could be obtained by coupling synthetic nitropyridinesulfenyl thioglycosides to a Fc unit engineered to have Cys-297 in lieu of glycan-linked Asn residue (Δh-Fc N297C). The considerable challenge of this endeavor would be the quantitative incorporation of not only a monosaccharide but also oligosaccharides of increasing complexity into a recombinant protein that has several disulfide linkages. Subsequent structural analysis should establish the site specificity of the conjugation, and biological experiments should determine whether the saccharide exerts any specific biological effects.

To this end, a range of thioaldoses (**6–9**) derived from *N*-linked glycan oligosaccharides (Figure 2) was prepared from the corresponding acetylated hemiacetal derivatives (**1–4**) by treatment first with a 1:2 mixture of thionyl chloride and toluene yielding the corresponding α-chlorides, which were subsequently taken up in acetone containing potassium thioacetate. The resulting β-thioacetate derivatives were treated with sodium methoxide in methanol to afford the *N*-glycan thioaldoses **6–9** in yields of 40%–70%. A maltopentaose thioaldose **10** was synthesized from peracetylated maltopentaose **5** for use as a control compound.

Although the nitropyridinesulfenyl thioglycoside of GlcNAc could easily be obtained from **6** [29], the preparation of similar derivatives of more complex sugars proved problematic. In a search for an alternative ap-

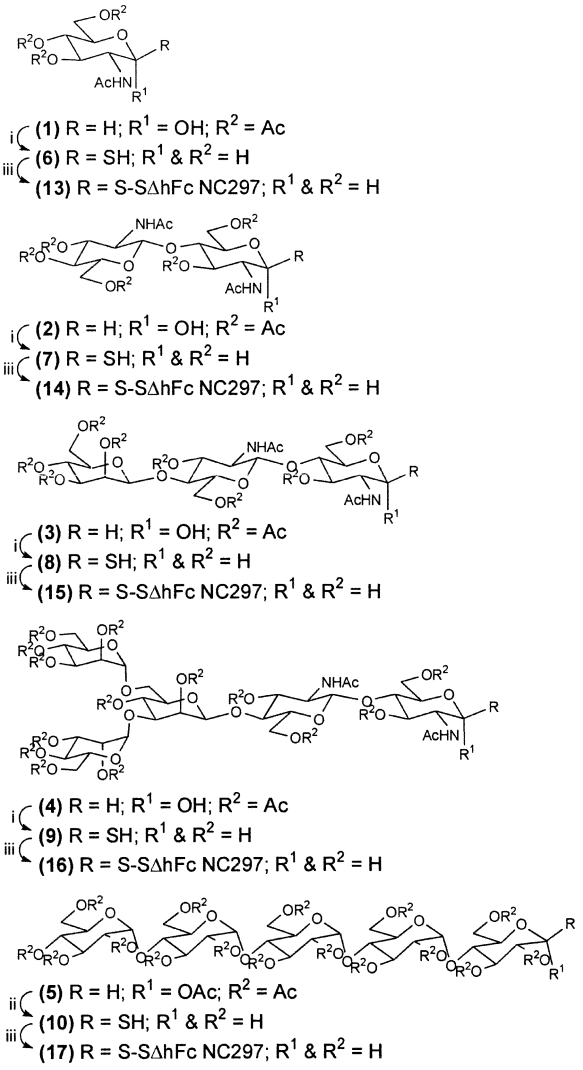

Figure 2. Glycosylation of a Hingeless Fc Cysteine Mutant with Thioaldoses

Conversion of peracetylated hemiacetals **1–5** to the corresponding thioaldoses **6–10** and their subsequent glycosylation of a hingeless Fc cysteine mutant (Δh-Fc N297C) to produce disulfide-linked Δh-Fc N297C-neoglycoconjugates **13–17**, respectively. Conditions: (i) (a) $SOCl_2$, (b) KSAc, (c) NaOMe/MeOH; (ii) (a) 30% w/v HBr in AcOH, (b) KSAc, (c) NaOMe/MeOH; (iii) 1% Δh-Fc N297C (w.r.t. thioaldose concentration), aqueous 12.5 mM ammonium acetate pH 8.5, 37°C.

proach to disulfide-linked neoglycoproteins, a direct coupling of a thioaldose (e.g., **6–10**) with a cysteine residue thiol group was considered. The aerial oxidation of thiols to disulfides is thought to occur via a free radical mechanism, where radicals generated from thiolates and super oxide form disulfides, but analogous thiol groups are less susceptible to free radical oxidation. Thus, it was expected that treatment of a cysteine-containing peptide or protein with an excess of a thioaldose at relatively high pH would lead to the formation of a mixed disulfide. Indeed, the combination of equimolar amounts of thioaldose **7** and reduced glutathione in aqueous 23 mM ammonium acetate at pH 8.5 yielded the disulfide-linked glycopeptide **11** (Figure 3), the thioaldose homodisulfide and glutathione disulfide in a statistical 2:1:1 ratio as determined by ^{1}H-NMR. When a larger excess of thioaldose **7** was used, only glycopeptide **11** and the sugar homodimer were present. Bovine serum albumin (BSA) was chosen as a model protein on the merit of its availability and convenience of having a single cysteine at position 58 of its protein sequence. After treatment of BSA with excess of **7** in aqueous ammonium acetate, the BSA-containing fractions were isolated free from excess **7** and other low molecular weight by-products by ultrafiltration (Figure 3). The excess of saccharide could be isolated as a disulfide from the filtrate by size exclusion column chromatography over P-2. Thiol concentration analysis of conjugate **12** using dithiodipyridine [33] gave a very low absorbance, indicating the absence of any free thiols, suggesting that the conjugation was quantitative. Further spectroscopic corroboration of conjugate **12** came from MALDI-TOF mass spectrometry, which showed a mass increase for conjugate **12** over BSA sample of 477 Da while the theoretical difference is 438 Da.

Having established a convenient procedure for the glycosylation of cysteine moieties of proteins, attention was focused on the glycosylation of an IgG-Fc fragment. Deleting the domains encoding for the Fab and hinge regions from the IgG genetic sequence created a truncated Fc unit glycoprotein (Δh-Fc). Further engineering to incorporate Cys-297 in place of the glycan binding Asn-297 gave aglycosylated cysteine mutant Δh-Fc N297C [34] (Figure 1). Epitope mapping using an extensive panel of monoclonal antibodies directed against the human IgG1 antibody revealed that neither the additional cysteine at residue 297 nor the absence of carbohydrate caused any large-scale structural changes including intradomain disulfide bond formation in the Δh-Fc N297C mutant, relative to Δh-Fc [34]. The rationale for engineering a hingeless Fc was to absent residues Cys-226 and Cys-229 within the hinge, which might otherwise compete with Cys-297 for glycosylation by thioaldoses. The Δh-Fc proteins were analyzed for purity and molecular composition by mass spectrometry and SDS-PAGE. Q-TOF mass spectrometry of Δh-Fc showed three peaks at 25,813; 25,976; and 26,138 Da (Table 1) corresponding to fucosylated glycoforms containing 0, 1, and 2 galactose residues (G0, G1, and G2), respectively. These average mass values are close to those calculated from the peptide sequence less the *N*-terminal lysine, which is lost during protein synthesis and processing [35], and formate incorporation, which can occur sometimes during protein mass spectroscopic analysis. As expected, SDS-PAGE of Δh-Fc showed a single band of 27 kDa, but Δh-Fc N297C produced two bands at 25 and 50 kDa (Figure 4) [34]. As only the 25 kDa band was visible when the disulfide bonds of Δh-Fc N297C were reduced with β-mercaptoethanol, it was deduced that the cysteine 297 had formed a disulfide linkage with its counterpart causing covalent dimerization of the two heavy chains. The dimerization was confirmed by MALDI-TOF mass spectrometry, displaying peaks at 24,456 and 48,913 corresponding to the mono- and dimeric forms of Δh-Fc N297C, respectively. The more exposed unnatural disulfide bond could be selectively reduced by treatment with mild reducing agent

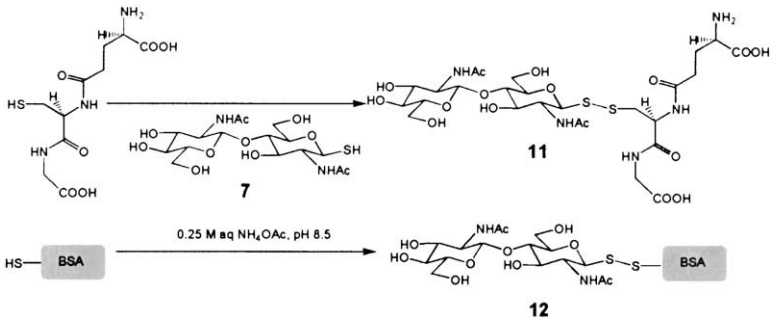

Figure 3. Preparation of Disulfide-Linked Neoglycopeptides and Neoglycoproteins

The coupling of thioaldose **7** with the cysteine thiol groups of glutathione and BSA to produce the corresponding disulfide-linked neoglycopeptide **11** and neoglycoprotein **12**, respectively.

cysteamine as determined by SDS-PAGE (Figure 4) and Q-TOF mass spectrometry (Table 1). Reaction of the resulting protein with thiochitobiose **7** afforded a product that had an increased mass of 412 Da, indicating that the Fc neoglycoconjugate **14** had been formed. Unfortunately, the yield of the conjugation was relatively low (~20%), and it was found that loss of protein was mainly due to the two purification steps required after reduction and glycosylation.

In an attempt to improve the conjugation yield, the unreduced protein was coupled directly with chitobiose thioaldose **7**. It was expected that in this reaction, a thiol disulfide interchange reaction between dimeric protein and excess **7** would give the expected product. Indeed, Q-TOF MS showed a peak at 24,594 Da correlating with the incorporation of a thiochitobiose moiety. The mass spectrum showed no peaks for the aglycosylated and additionally glycosylated proteins, indicating that the conjugation had gone to completion and that only one saccharide moiety had been incorporated for each heavy chain fragment. Gratifyingly, the direct thioaldose glycosylation resulted in a greatly improved yield of 60% of the isolated neoglycoconjugate **14**.

As Δh-Fc N297C contains cystine disulfide bridges C261–C321 and C367–C425, it could not be established at this point whether these cystine groups had been glycosylated by **7** or had been formed correctly in the first place. To address this important issue, the chitobiose glycoconjugate **14** was subjected to trypsin digestion and the resulting peptide fragments analyzed by MALDI-TOF mass spectrometry. Detection of a peak at 1618.1 Da corresponding to the glycosylated peptide of the predicted cleavage peptide (293–301) EEQYCSTYR of mass 1178.5 Da + thiochitobiose (1616.6 Da) confirmed that glycosylation had occurred at the correct position. Furthermore, a peak at 2332.1 Da correlating to the predicted peptide fragment containing the C261–C321 cystine-bridge (256–274 + 321–322) TPEVTC(CK)VVVDVSHEDPEVK of mass 2330.2 Da, which indicated that this disulfide bond had remained intact during glycosylation. Although no peak could be found corresponding to the peptide fragment containing the C367–C425 cysteine bridge, the absence of peaks corresponding to glycosylated fragments containing C367 and C425 indicated that no unspecific glycosylation had occurred. In addition, tryptic digest of Δh-Fc did also not show the C261–C321 cystine bridge or corresponding reduced peptides. Selective reduction and alkylation of Δh-Fc N297C, followed by trypsin digestion produced a peak at 1236.2 Da corresponding to alkylated peptide fragment (293–301) EEQYCSTYR of mass 1178.5 Da + acetamide (1235.5 Da).

Having established an efficient conjugation approach, Δh-Fc N297C was treated with thioaldoses **6**, **8**, **9**, and

Table 1. Mass-Spectral Analysis of Δh-Fc Proteins: Δh-Fc, Δh-Fc N297C, and Homogeneous Δh-Fc-Neoglycoconjugates **13–17**

Δh-Fc-Protein	MALDI-TOF Mass (Da)	Q-TOF Mass (Da)	Calculated Mass[c] (Da)
Δh-Fc N297C	24,456; 48,913[a]	24,356	24,356
Δh-Fc N297C reduced	24,478	24,431[b]	24,432
+[-SGlcNAc] **13**	24,693	24,593	24,591
+[-S(GlcNAc)$_2$] **14**	24,868	24,793	24,794
+[-S(GlcNAc)$_2$Man] **15**	25,030	—	24,956
+[-S(GlcNAc)$_2$Man$_3$] **16**	25,393	—	25,281
+[-SGlc$_5$] **17**	25,213	—	25,198
Δh-Fc[d]	—	25,813[e]	25,812
		25,976[f]	25,974
		26,138[g]	26,136

ExPASy-calculated average molecular weight of aglycosylated Δh-Fc peptide sequence, less N-terminal lysine (−128.2 Da) and incorporation of formate (+27 Da) = 24366.5.

[a] Observed mass of dimer protein.

[b] Δh-Fc N297C coupled with cysteamine (+76 Da).

[c] ExPASy calculated average molecular weight of peptide sequence, less N-terminal lysine (−128.2 Da) and incorporation of formate (+27 Da).

[d] Glycan is fucosylated N-glycan octasaccharide.

[e] N-glycan octasaccharide with 0 Gal residues.

[f] N-glycan octasaccharide with 1 Gal residue.

[g] N-glycan octasaccharide with 2 Gal residues.

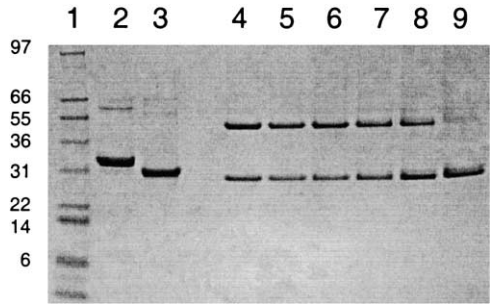

Figure 4. Reduction of Δh-Fc N297C with Mild Reducing Agent Cysteamine

SDS-PAGE of Δh-Fc (lane 2) and Δh-Fc N297C (lane 3) reduced with β-mercaptoethanol and unreduced Δh-Fc N297C (lane 4). Incubations of Δh-Fc N297C with mild reducing agent cysteamine at concentrations 0, 0.02, 0.2, 2, and 20 mM are shown in lanes 5–9, respectively.

10 (Figure 2). In each case, mass spectrometry of the purified conjugates **13**, **15**, **16**, and **17**, respectively, showed the expected increase in mass (Table 1). The successful preparation of these neoglycoproteins demonstrate for the first time that by in vitro glycosylation, complex oligosaccharides can be incorporated in a site-specific manner into a recombinant protein that has its natural *N*-linked glycosylation site replaced by cysteine. Previously, the site-specific glycosylation of protein cysteines only involved monosaccharides [30, 36] and the more simple chitotriose [37].

The Δh-Fc N297C neoglycoproteins **13–17** were examined for their ability to interact with human FcγRI present on U937 leukocyte cells and the results were compared to Δh-Fc. A 2 day incubation of U937 cells with γ-interferon enhances cell-surface expression of FcγRI required for the superoxide burst elicited by U937 cells upon interaction with IgG1-sensitized JY cells. The interaction of FcγRI with the Fc regions of the IgG antibodies present on the JY cells triggers the superoxide burst, which can be inhibited by IgG and by Fc. As expected, effective inhibition of the superoxide burst was observed for the glycosylated Δh-Fc (Figure 5). The aglycosylated Δh-Fc N297C was found to be an almost 100-fold worse inhibitor, demonstrating that glycosylation is of critical importance for FcγRI binding. The neoglycoprotein **13**, having a disulfide-linked *N*-acetyl glucosamine moiety showed no improvement in inhibitory activity; however, **14–16**, having a di-, tri-, or pentasaccharide attachment, respectively, were markedly better inhibitors. Furthermore, the activity improved with increasing complexity of the attached carbohydrate. To demonstrate that the enhanced biological properties of the neoglycoconjugates were not the result of indirect steric effects attributed to the bulk of the attached sugar, maltopentose thioaldose **10** was conjugated to Δh-Fc N297C to afford neoglycoprotein **17**. Although glycosylation of Δh-Fc N297C with **10** produced slightly higher superoxide inhibition, it displayed much poorer inhibition than the pentasaccharide neoglycoprotein **16**, demonstrating the importance of the nature of the glycan structure (Figure 5). The pentasaccharide neoglyconjugate **16** was, however, not as active as the wild-type glycosylated Δh-Fc. The latter glycoprotein contains oligosaccharides that are much more complex, possessing between 8 and 12 monosaccharide residues with the majority having a fucoside and one or two galactosides in addition to the outer-arm *N*-acetyl glucosamine residues. It is well known that the presence of these residues improve IgG effector functions, and for example, it has been shown galactosylation of the outer-arm GlcNAc residues accounts for significant increases in FcγRI activity [10, 38] and the effect of outer-arm sialylation may either be additive or synergistic [39]. Thus, it is to be expected that the attachment of more complex oligosaccharides to Δh-Fc N297C will lead to conjugates with further improved biological activities.

The in vitro glycosylation technology described here offers several distinct advantages for the preparation of well-defined glycoforms of glycoproteins. In particular, synthetic oligosaccharides can be incorporated that are of low natural abundance but which are known to display exceptional bioactivities. For example, IgG glycoforms that possess a bisecting GlcNAc moiety have a 50-fold increased FcγRIII activity but in general are present in very low natural abundance [40]. Thus, even in the case that neoglycoconjugation will produce glycoproteins that are not as active as their natural-linked counter parts, improved bioactivities can be obtained by attachment of oligosaccharides that are known to induce high biological activities. In vitro glycosylation can provide quantitative incorporation of oligosaccharides, whereas

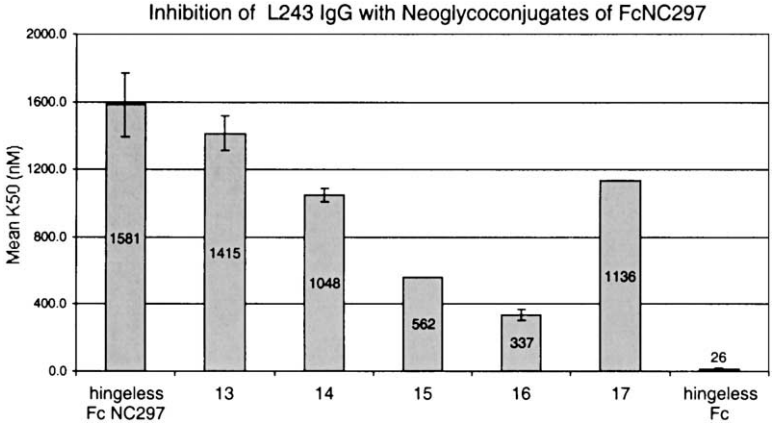

Figure 5. Inhibition of Superoxide Production with Glycosylated, Aglycosylated, and Neoglycosylated Δh-Fc

Inhibition of superoxide production by U937 cells triggered by IgG1-sensitized JY cells with glycosylated, aglycosylated, and neoglycosylated Δh-Fc: Δh-Fc, Δh-Fc N297C and homogeneous Δh-Fc-neoglycoconjugates **13–17**. Superoxide inhibition is represented as the K_{50} inhibitory value in nanomolars for each Fc-protein, whereby the average has been calculated (SEM given for n = 3).

variable and fractional occupancies may be observed in glycoproteins obtained by cell culture. For example, recombinant IgG proteins and their truncated forms produced by mammalian cells can in some cases have as little as one-third of their heavy chain Asn-297 residues glycosylated [31, 34]. In vitro glycosylation allows more efficient bacterial or yeast protein expression systems to be used, which due to lack of protein glycosylation and glycan remodeling, respectively, are not applicable to the production of mammalian glycoproteins. Also, the incorporation of unnatural oligosaccharide residues will be possible which may lead to neoglycoconjugates that have improved biostabilities. A synthetic strategy will also make it possible to attach toxins or other reagents to provide neoglycoproteins with novel effector functions.

Significance

Mammalian glycoprotein biosynthesis produces a heterogeneous range of proteins that possess the same peptide backbone but differ in the nature and site of glycosylation. This feature has frustrated efforts to elucidate biological functions of glycoproteins, in particular in those cases in which the precise structure of a glycan determines biological activity. Furthermore, the inability to control *N*-glycan formation in cell culture is a major obstacle in the development of therapeutic glycoproteins. Although chemists have made good progress toward the chemical synthesis of native well-defined glycopeptides, the preparation of even a small glycoprotein is a formidable task. We have demonstrated that a recombinant protein that has its natural *N*-linked glycosylation site replaced by cysteine can be glycosylated with complex synthetic oligosaccharides to give disulfide-linked glycoproteins. This technology was applied to the glycosylation of an IgG1-Fc fragment, engineered to have Cys-297 in place of glycan-linked Asn, where mass spectrometric analysis combined with protease digestion indicated that oligosaccharides as complex as a pentasaccharide could be attached in a site-specific manner. The resulting neoglycoproteins were tested for their ability to interact with human FcγRI by inhibiting superoxide production by γ-interferon-stimulated U937 cells. These studies showed for the first time that the biological activity of a glycoprotein lost by removal of its natural glycosylation site can be restored partially by the in vitro attachment of synthetic saccharides. Although the neoglycoconjugates were not as active as the corresponding *N*-linked glycoprotein, it is to be expected that further improvements of activity can be obtained be employing more complex oligosaccharides. In vitro glycosylation of recombinant proteins offers several distinct advantages, including quantitative conjugation of oligosaccharides, the incorporation of oligosaccharides that are of low natural abundance but which are known to display high bioactivities, and the possibility of using more efficient bacterial or yeast protein expression systems.

Experimental Procedures

General Experimental Details

Chemicals were purchased from Acros (Pittsburgh, PA) or Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), and Fluka (Milwaukee, WI). Protein concentrations were determined using a Lowery protein concentration kit from Sigma, where UV absorption was recorded at 690 nm using a Finstruments 96-well ELISA-plate reader. Thiol concentrations were determined using purified *S*,*S*-dithiodipyridine as described by Grassetti and Murray [33], and the absorption was recorded at 340 nm using a Finstruments ELISA-plate reader. Mass spectra were obtained using a Micromass Q-TOF-2 mass spectrometer and a Hewlett Packard G2025A MALDI-TOF mass spectrometer in positive mode using sinapinic acid as the matrix unless stated otherwise. High-resolution mass spectra were obtained using a Voyager delayed extraction STR with 2,5-dihydroxybenzoic acid as an internal calibration matrix. Construction of the pEE14 Δh-Fc and pEE14 Δh-FcNC297 expression vectors and their transfection into CHO-K1 cells has been described previously [34]. The chemical synthesis of the *N*-glycan core tri- and pentasaccharide structures will be described elsewhere. 2-acetamido-2-deoxy-β-D-glucopyrano-thioaldose **6** was synthesized as described previously [29].

2-Acetamido-2-Deoxy-β-D-Glucopyranosyl-(1-4)-2-Acetamido-2-Deoxy-β-D-Glucopyrano-Thioaldose (7)

A stirred suspension of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-glucopyranose **2** (100 mg, 0.16 mmol) in a 1:1 mixture of anhydrous DCM and anhydrous toluene (1 ml) was treated with thionyl chloride (1.5 ml) at room temperature for 2 hr. Removal of the solvent and reagent under reduced pressure gave the α-chloride derivative, which was subsequently taken up in acetone (6 ml) containing potassium thioacetate (26 mg, 0.23 mmol). The resulting mixture was stirred at room temperature for 18 hr, and the solvent was removed under reduced pressure. Flash-column chromatography of the residue using a 2%–6% gradient of methanol in DCM afforded the peracetylated thioaldose (44 mg), which was treated with a 0.5 M solution sodium methoxide in methanol (5 ml) for 30 min. After neutralization of the reaction mixture using Amberlite 650C ion exchange resin, the solvent was removed in vacuo to give *the title compound* **7** (28 mg, 40%) as a white solid; ^{1}H NMR (D_2O, 600 MHz) δ 4.53 (d, 1H, $J_{1\text{-}2}$ = 10.3 Hz, H-1), 4.44 (d, 1H, $J_{1'\text{-}2'}$ 8.3 Hz, H-1′), 3.77 (dd, 1H, $J_{5'\text{-}6b'}$ 1.9, $J_{6a'\text{-}6b'}$ 12.2 Hz, H-6b′), 3.68 (dd, 1H, $J_{5\text{-}6b}$ 2.0, $J_{6a\text{-}6b}$ 12.2 Hz, H-6b), 3.64 (t, 1H, *J* 9.8 Hz, H-2), 3.60 (dd, 1H, $J_{5'\text{-}6a'}$ 5.4 Hz, H-6a′), 3.60 (dd, 1H, $J_{2'\text{-}3'}$ 10.3 Hz, H-2′), 3.52 (dd, 1H, $J_{2\text{-}3}$ 9.7, $J_{3\text{-}4}$ 8.8 Hz, H-3), 3.49 (m, 1H, H-4), 3.49 (dd, 1H, $J_{5\text{-}6a}$ 5.4 Hz, H-6a), 3.42 (dd, 1H, $J_{3'\text{-}4'}$ 8.3 Hz, H-3′), 3.38 (m, 1H, H-5), 3.34 (m, 1H, H-5′), 3.32 (dd, 1H, $J_{4'\text{-}5'}$ 9.8 Hz, H-4′), 1.92 and 1.89 (2 × s, 6H, 2 × $COCH_3$); MALDI-TOF MS (with 2,5-dihydroxybenzoic acid as matrix): 461.9 ($M+Na^+$) and 477.9 ($M+K^+$); HR MS (m/z): calcd for $C_{16}H_{28}N_2NaO_{10}S$ 463.1337, found 463.1362.

β-D-Mannopyranosyl-(1-4)-2-Acetamido-2-Deoxy-β-D-Glucopyranosyl-(1-4)-2-Acetamido-2-Deoxy-β-D-Glucopyrano-Thioaldose (8)

2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl-(1-4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-glucopyranose **3** (52 mg, 54 μmol) was subjected to identical experimental conditions as for compound **7** to give *the title compound* **8** (22 mg) in 41% yield; ^{1}H NMR (D_2O, 500 MHz) δ 4.60 (s, 1H, H-1″), 4.50 (d, 1H, $J_{1\text{-}2}$ 10.3 Hz, H-1), 4.42 (d, 1H, $J_{1'\text{-}2'}$ 7.6 Hz, H-1′), 3.88 (d, 1H, $J_{2''\text{-}3''}$ 3.4 Hz, H-2″), 3.81 (dd, 1H, $J_{5''\text{-}6b''}$ 2.0, $J_{6a''\text{-}6b''}$ 12.2 Hz, H-6b″), 3.77 (dd, 1H, $J_{5'\text{-}6b'}$ 2.0, $J_{6a'\text{-}6b'}$ 12.3 Hz, H-6b′), 3.71 (dd, 1H, $J_{5\text{-}6b}$ 2.0, $J_{6a\text{-}6b}$ 12.3 Hz, H-6b), 3.69–362 (m, 5H, H-2, H-2′, H-3′, H-4′ and H-6a′), 3.61 (dd, 1H, $J_{5''\text{-}6a''}$ 6.8 Hz, H-6a″), 3.55–3.50 (m, 4H, H-3, H-4, H-6a and H-3″), 3.50 (m, 1H, H-5′), 3.45 (dd, 1H, $J_{3''\text{-}4''}$ 9.3, $J_{4''\text{-}5''}$ 9.8 Hz, H-4″), 3.34 (m, 1H, H-5), 3.30 (m, 1H, H-5″), 1.94 and 1.93 (2 × s, 6H, 2 × $COCH_3$); MALDI-TOF MS (with 2,5-dihydroxybenzoic acid as matrix): 624.7 ($M+Na^+$) and 640.7 ($M+K^+$); HR MS (m/z): calcd for $C_{22}H_{38}N_2NaO_{15}S$ 625.1855, found 625.1891.

α-D-Mannopyranosyl-(1-6)[α-D-mannopyranosyl-(1-3)]-β-D-Mannopyranosyl-(1-4)-2-Acetamido-2-Deoxy-β-D-Glucopyranosyl-(1-4)-2-Acetamido-2-Deoxy-β-D-Glucopyrano-Thioaldose (9)

2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1-6)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1-3)-]-2,4-di-*O*-acetyl-β-D-mannopyranosyl-(1-4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-D-glucopyranose **4** (38

mg, 25.6 μmol) was subjected to identical experimental conditions as for compound **7** to give *the title compound* **9** (16 mg, 41%) as a white solid; ^{1}H NMR (D_2O, 500 MHz) δ 4.92 (s, 1H, H-1‴), 4.72 (s, 1H, H-1⁗), 4.61 (s, 1H, H-1″), 4.52 (d, 1H, $J_{1\text{-}2}$ 11.0 Hz, H-1), 4.44 (d, 1H, $J_{1'\text{-}2'}$ 7.7 Hz, H-1′), 4.08 (d, 1H, $J_{2''\text{-}3''}$ 3.4 Hz, H-2″), 3.89 (m, 1H, H-2‴), 3.79 (m, 1H, H-2⁗), 3.78–3.66 (m, 8H), 3.66–3.51 (m, 10H), 3.51–3.42 (m, 6H), 3.42–3.30 (m, 3H), 1.92 and 1.89 (2 × s, 6H, 2 × $COCH_3$); MALDI-TOF MS (with 2,5-dihydroxybenzoic acid as matrix): 949.9 ($M+Na^+$) and 965.8 ($M+K^+$); HR MS (m/z): calcd for $C_{34}H_{58}N_2NaO_{25}S$ 949.2898, found 949.2947.

α-D-Glucopyranosyl-(1-4)-α-D-Glucopyranosyl-(1-4)-α-D-Glucopyranosyl-(1-4)-α-D-Glucopyranosyl-(1-4)-β-D-Glucopyrano-Thioaldose (10)

Peracetylated maltopentaose **5** (36 mg, 24 μmol) in DCM (0.6 ml) was treated with a 30% solution of HBr in acetic acid (0.5 ml) for 4 hr before dilution with DCM (15 ml) washing in-turn with ice/water (10 ml), saturated solution of sodium bicarbonate (10 ml) and ice/water (10 ml), drying ($MgSO_4$), and removal of the solvents under reduced pressure. The resulting glycosyl bromide (26 mg, 17 μmol) was treated with acetone (2 ml) containing potassium thioacetate (2.5 mg, 22.1 μmol), and the resulting mixture was stirred at room temperature for 18 hr; thereafter, the solvent was removed under reduced pressure. Flash-column chromatography (gradient of 2%–6% methanol in DCM) of the residue afforded the peracetylated thioaldose, which was deacetylated as described above to give *the title compound* **10** (21 mg, 80%) as a white solid; ^{1}H NMR (D_2O, 500 MHz) δ 5.29 (m, 4H, H-1′, H-1″, H-1‴ and H-1⁗), 4.46 (d, 1H, $J_{1\text{-}2}$ 9.3 Hz, H-1), 3.87–3.79 (m, 3H), 3.79–3.68 (m, 10H), 3.68–3.45 (m, 15H), 3.31 (dd, 1H, $J_{2\text{-}3}$ 9.3, $J_{3\text{-}4}$ 9.8 Hz, H-3) and 3.18 (t, 1H, *J* 9.3 Hz, H-2); MALDI-TOF MS (with 2,5-dihydroxybenzoic acid as matrix): 844.6 ($M+Na^+$) and 860.5 ($M+K^+$); HR MS (m/z): calcd for $C_{34}H_{58}N_2NaO_{25}S$ 867.2386, found 867.2416.

2-Acetamido-2-Deoxy-β-D-Glucopyranosyl-(1-4)-2-Acetamido-2-Deoxy-1-Thio-β-D-Glucopyrano-*S*′-*S*-Glutathione Disulfide (11)

A solution (20 ml) of 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-2-deoxy-β-D-glucopyrano-thioaldose **7** (5 mg, 11.0 μM) and glutathione (1.7 mg, 5.7 μM) in aqueous 25 mM ammonium acetate at pH 8.5 was stirred at room temperature for 18 hr. The reaction mixture was freeze-dried and *the disulfide-linked glycopeptide* **11** (3.2 mg, 76%) was isolated by P-2 gel-filtration (water as eluent). ^{1}H NMR (D_2O, 500 MHz) δ 4.80 (dd, 1H, *J* 3.9 and 9.8 Hz, CH-Cys), 4.62 (d, 1H, $J_{1,2}$ 10.7 Hz, H-1), 4.58 (d, 1H, $J_{1',2'}$ 8.9 Hz, H-1′), 4.04 (dd, 1H, $J_{2,3}$ 9.8 Hz, H-2), 3.91 (dd, 1H, $J_{5',6b'}$ 1.9, $J_{6a',6b'}$ 11.7 Hz, H-6b′), 3.86 (dd, 1H, $J_{5,6b}$ 1.9, $J_{6a,6b}$ 11.7, H-6b), 3.69–3.81 (m, 6H, CH_2-Gly, CH-Glu, H-2′, H-3, and H-6a′), 3.66 (dd, 1H, $J_{5,6a}$ 5.9 Hz, H-6a), 3.65 (dd, 1H, $J_{3,4}$ 8.8, $J_{4,5}$ 9.8 Hz, H-4), 3.55 (dd, 1H, $J_{2',3'}$ 10.7, $J_{3',4'}$ 8.8 Hz, H-3′), 3.54 (m, 1H, H-5), 3.50 (m, 1H, H-5′), 3.46 (dd, 1H, $J_{4',5'}$ 9.7 Hz, H-4′), 3.36 (dd, 1H, *J* 3.9 and 14.6 Hz, CH_2-Cys), 2.99 (dd, 1H, *J* 9.8 and 14.6 Hz, CH_2-Cys), 2.52 and 2.15 (2 × m, 4H, 2 × CH_2-Glu), 2.06 and 2.01 (2 × s, 6H, 2 × CH_3CO); MALDI-TOF MS (+ve): 746.2 ($M+H^+$), 768.2 ($M+Na^+$), 784.2 ($M+K^+$), and 789.2 ($M\text{-}H^+ + 2Na^+$).

2-Acetamido-2-Deoxy-β-D-Glucopyranosyl-(1-4)-2-Acetamido-2-Deoxy-1-Thio-β-D-Glucopyrano-*S*′,*S*-BSA Disulfide (12)

A solution of BSA (15 μM) and 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-2-deoxy-β-D-glucopyrano-thioaldose **7** (750 μM) in 0.25 M ammonium acetate at pH 8.5 (1 ml) was stirred at 30°C. After 24 hr the free thiol concentration, determined using *S*,*S*-dithiodipyridine, was very low. An aliquot (500 μl) was removed and subjected to ultra filtration using Millipore Centricon devices fitted with 10 kDa cutoff membranes. The concentrate was washed three times with water (500 μl) and taken up in water (300 μl) to give neoglycoprotein **12**, which was subjected to MALDI-TOF MS; **12** = 67,089 Da (BSA = 66,612 Da).

Selective Reduction of Δh-Fc N297C

A 1 mg/ml solution of Δh-Fc N297C in PBS was treated with cysteamine (final concentration 20 mM) at 37°C. After 30 min the cysteamine was removed by microdialysis using a Pierce Microdialysis device fitted with a 10 kDa cutoff membrane in water for 1 hr. The resulting Δh-Fc N297C bearing reduced cysteines at the point of mutation was immediately incubated with thioaldose (50 equivalents) as described below. Alternatively, for analytical purposes, the selectively reduced Δh-Fc N297C was treated with iodoacetamide (final concentration 40 mM) to quench any remaining cysteamine and to alkylate the cysteine groups preventing reformation of the undesired disulfide bond. The alkylated protein was subjected to SDS-PAGE and trypsin digestion as described below for compound **14**.

Formation of of Δh-Fc N297C Neoglycoproteins (13–17)

To a 20 nM solution of Δh-Fc N297C in PBS (50 μl) was added aqueous thioaldose (50 equivalents) and 0.25 M ammonium acetate at pH 8.5 (8 μl). The resulting mixture was incubated with occasional agitation at 37°C for 5 hr, whereupon a second portion of the thioaldose (50 equivalents) solution was added. After 16 hr at 37°C, the mixture was subjected to microdialysis against water for 2 hr using Pierce Microdialysis devices fitted with 10 kDa cutoff membranes. The neoglycoconjugates were analyzed by MALDI-TOF MS and Q-TOF MS and their concentrations determined using the Lowery assay [41].

Trypsin Digest of Chitobiose Fc-Neoglycoprotein (14)

The chitobiose neoglycoconjugate **14** (20 nM) in PBS (25 μl) was subjected to microdialysis against 25 mM ammonium acetate (pH 7.4) for 1 hr using a Pierce Microdialysis device fitted with a 10 kDa cutoff membrane. The resulting neoglycoconjugate solution was treated with a solution of sequencing grade trypsin from Sigma (1 mg/ml) in 1 mM HCl (1 μl) at 37°C for 24 hr, whereupon the mixture was subjected to MALDI-TOF MS using α-cyano-4-hyroxy cinnamic acid as the matrix. A 20 nM solution of the selectively reduced and alkylated Δh-Fc N297C subjected to identical sequencing conditions was used as a control.

Superoxide Burst Inhibition Assay

U937 cells were pretreated with χ-IFN (1000 U/ml) for 2 days; thereafter, the cells were washed and resuspended at 2 × 10^6 cells/ml in Hanks balanced salt solution (HBSS) containing BSA (15 μM). Target JY cells both lacking (baseline control) and sensitized with a humanized IgG1 (20 nM) were washed and resuspended at 2 × 10^7 cells/ml in HBSS/BSA. U937 cells (2 × 10^5 cells/ml), the appropriate concentration of inhibitor (0.2–200 nM for Δh-Fc and 2.0–2000 nM for Δh-Fc N297C and neoglycoconjugates **13–17**) and lucigenin (250 μM) were incubated for 5 min at 37°C. JY cells were added (2 × 10^6 cells/ml) to initiate the assay and superoxide production was measured as lucigenin-enhanced chemiluminecence for 30 min at 37°C using a Berthold LB953 luminometer [34]. The chemiluminecence response curve area values were plotted against inhibitor concentration using Microsoft Excel to create an inhibition curve from which the inhibition constant, IC_{50} was obtained. Assays were performed in triplicate and the IC_{50} results averaged.

Supplemental Data

Supplemental data include ^{1}H-NMR spectra of thioaldoses **2–5**, Q-Tof mass spectra of Δh-Fc, Δh-Fc NC297, Δh-Fc NC297 (reduced with cysteamine), and Δh-Fc NC297 neoglycoconjugates **8** and **9**, and superoxide inhibition curves for Δh Fc NC297 and neoglycoconjugates **8–11**. The Supplemental Data is available at http://www.chembiol.com/cgi/content/full/10/9/807/DC1.

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