Metabolic Functionalization of Recombinant Glycoproteins†

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ABSTRACT: Glycoproteins are essential for cellular communication and are the most rapidly growing class of therapeutic agents. Chemical modification of glycoproteins has been employed to improve their in vivo efficacy or to label them for detection. Methods for the controlled derivatization of glycoproteins are presently limited by the repertoire of natural amino acid side chain and carbohydrate functionalities. Here, we use metabolic oligosaccharide engineering to introduce a bioorthogonal functional group, the azide, into cellular and recombinant glycoproteins for subsequent chemical elaboration via Staudinger ligation. As most therapeutic glycoproteins are sialylated and require this saccharide for optimal pharmacokinetics, we targeted sialic acid as a host for azides using *N*-azidoacetylmannosamine (ManNAz) as a biosynthetic precursor. Metabolic conversion of ManNAz to *N*-azidoacetylsialic acid (SiaNAz) within membrane-bound and secreted glycoproteins was quantified in a variety of cell types. SiaNAz was found to comprise between 4% and 41% of total sialosides, depending on the system. Metabolic labeling of recombinant interferon-β and GlyCAM-Ig was achieved, demonstrating the utility of the method for functionalizing N-linked and O-linked glycoproteins of therapeutic interest. More generally, the generation of recombinant glycoproteins containing chemical handles within their glycans provides a means for studying their behavior and for improving their in vivo efficacy.

As modulators of the inflammatory response (*1*, *2*) and regulators of a number of cellular processes (*3*−*6*), glycoproteins are vital for cellular communication and have become increasingly important as therapeutic agents (*7*, *8*). The functions of many glycoproteins are now well understood, and it is clear that their glycans can be important determinants of biological activity. In many cases, the protein component encodes the fundamental bioactivity while the glycans serve to target the molecule to specific tissues, to improve stability or to govern serum half-life. In other cases, the glycans provide epitopes for biorecognition that determine the mechanism of action. Because of the importance of glycoproteins as therapeutic agents, there is a high demand for methods that improve their properties and tools for determining the roles of their glycans (*8*).

A number of approaches to engineering glycoproteins with enhanced properties have been successfully employed in a therapeutic setting (*9*, *10*). For example, the serum half-life of erythropoietin (EPO) is largely dependent on its glycosylation state, and likewise, enhancement of its glycan structures via engineered cell lines has been widely explored as a means to improve in vivo efficacy (*10*−*12*). Alternatively, the pharmacokinetic properties of EPO have been improved by chemical conjugation of the protein with poly(ethylene glycol) (PEG)[1] (*13*), a modification that generally increases the serum half-life of recombinant proteins (*9*, *14*). Other glycoproteins have been chemically modified by oxidation of their glycans to introduce aldehydes followed by selective conjugation reactions, although certain amino acid residues are damaged in this procedure (*15*, *16*). Genetic strategies include dimerization or fusion to heterologous protein domains such as IgG heavy chains, which confer excellent pharmacokinetic properties (*17*−*20*). These examples demonstrate the range of modifications to glycoprotein structures that can be achieved using the chemical reactivity of their natural constituents. The ability to augment recombinant glycoprotein structures using functionality not found in nature offers the potential for even more dramatic improvements to their biological properties.

We have been generally interested in the use of bioorthogonal chemical ligations (i.e., ligations in which two

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[1] Abbreviations: PEG, poly(ethylene glycol); NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; SiaNAz, *N*-azidoacetylsialic acid; IFN-β, interferon-β; GlyCAM-Ig, GlyCAM-1 fused to the Fc portion of a human immunoglobulin G; ManNAc, *N*-acetylmannosamine; C2GnT, core 2 *N*-acetylglucosaminyltransferase; FucT7, fucosyltransferase VII; ManNAz, *N*-azidoacetylmannosamine; Ac_4ManNAz, peracetylated *N*-azidoacetylmannosamine; CHO, Chinese hamster ovary; FLAG, DYKDDDDK peptide; PCR, polymerase chain reaction; DMB, 1,2-diamino-4,5-methylenedioxybenzene.

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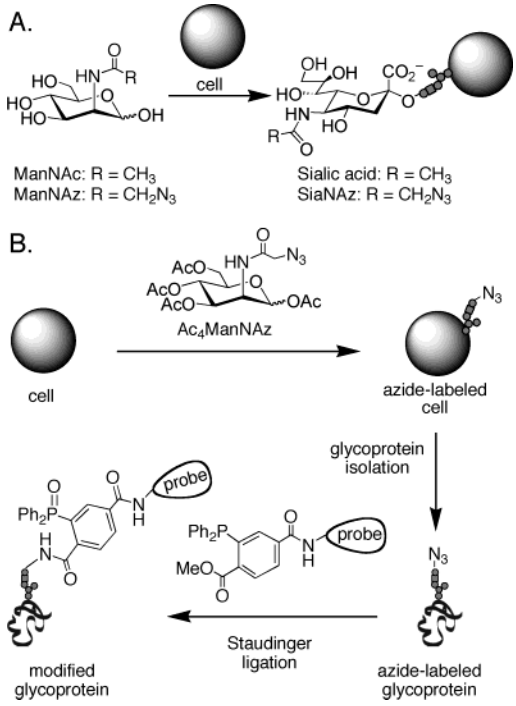

FIGURE 1: Metabolic oligosaccharide engineering as a means to label glycoconjugates with bioorthogonal functional groups. (A) ManNAc and its unnatural analogue ManNAz are converted into the corresponding sialic acids, which are incorporated into cell surface glycoconjugates. (B) Metabolic labeling of glycoproteins by expression in the presence of Ac_4ManNAz primes them for further modification by Staudinger ligation. The peracetylated sugar permeates cell membranes more readily than the free sugar and can therefore be used at lower concentrations (*53*, *54*).

functional groups react exclusively with each other in a biological milieu) as a means to augment the structures of biopolymers produced by cells (*21*). This objective requires methods for introducing bioorthogonal functional groups into recombinant proteins or polysaccharides using the cell's metabolic machinery. In the case of proteins, this can be accomplished using unnatural amino acids and cellular enzymes that permit their residue-specific or site-specific incorporation into recombinant proteins (*22*−*27*). Although these techniques are now well established in yeast and bacterial expression systems, their application to mammalian glycoproteins is limited. The introduction of bioorthogonal functional groups into mammalian glycoproteins will therefore require alternative strategies.

Given that glycoproteins comprise two biopolymers, proteins and polysaccharides, we have focused on the latter as hosts for bioorthogonal functionality. Previous work in our laboratory and others has demonstrated that unnatural monosaccharides bearing bioorthogonal functional groups can be metabolized and incorporated into mammalian cell surface glycans (*28*−*33*). For example, an azide-modified derivative of *N*-acetylmannosamine (ManNAc) termed *N*-azidoacetylmannosamine (ManNAz, Figure 1A) is metabolized and incorporated into cell surface glycoconjugates, presumably as the corresponding sialic acid (SiaNAz). The azide can be further elaborated by Staudinger ligation with phosphine probes (*30*), generating a covalent adduct on the cell surface between the glycoconjugate and the exogenous reagent. This method, which we term metabolic oligosaccharide engineering, has been applied to a number of fundamental studies in glycobiology (*34*−*36*). Here, we report the extension of the technology to engineer recombinant glycoproteins for selective covalent modification. As shown in Figure 1B, glycoproteins expressed by cells treated with a peracetylated form of ManNAz (Ac_4ManNAz) can be isolated and the azido groups pendant on their glycans further elaborated with exogenous phosphine reagents. The Staudinger ligation is completely bioorthogonal and therefore does no damage to the underlying protein or glycan. The approach was applied to the chemical modification of membrane-associated molecules and overexpressed recombinant glycoproteins of therapeutic interest.

MATERIALS AND METHODS

Materials. HiTrap chelating columns were from Amersham Biosciences. T4 DNA ligase and all restriction enzymes were from New England Biolabs. RPMI-1640, HAM F-12 nutrient mixture, Dulbecco's minimal essential media (DMEM), OptiMEM, the vector pSecTag2A, and Lipofectamine Plus were from Invitrogen Life Technologies. Centricon filters (3000 molecular weight cutoff) were from Amicon. FITC−avidin, penicillin, streptomycin, and peroxidase-conjugated anti-FLAG M2 monoclonal antibody (anti-FLAG-HRP) were from Sigma. Fetal calf serum (FCS) was from Hyclone. Cell densities were determined using a Coulter Z2 cell counter. Peroxidase-conjugated anti-mouse IgG monoclonal (anti-mouse-HRP) and anti-human IgG monoclonal (anti-IgG-HRP) antibodies were from Jackson Immunoresearch Laboratories. Unconjugated mouse anti-c-Myc (9E10) monoclonal antibody was from Santa Cruz Biotechnology. The SuperSignal West Pico chemiluminescent substrate was from Pierce. The gene encoding human interferon-β (IFN-β) was obtained from the ATCC (no. 31902) encoded in the vector pLG104R. Protein A−Sepharose 4B was from Zymed. Trypsin gold, mass spectrometry grade, was obtained from Promega, and *Vibrio cholerae* sialidase was from Calbiochem. The vectors encoding GlyCAM-1 fused to the human IgG heavy chain (GlyCAM-Ig), core 2 GlcNAc transferase (C2GnT), and fucosyltransferase VII (FucT7) were a generous gift from Steven Rosen (University of California, San Francisco). Ac_4ManNAz (*30*) and SiaNAz (*37*) were synthesized as previously described.

Cell Culture Conditions. All cell lines were maintained in a 5% CO_2, water-saturated atmosphere at 37 °C, and media were supplemented with penicillin (100 unit/mL), streptomycin (0.1 mg/mL), and 10% FCS. Jurkat cells, a human T-cell lymphoma line, were maintained in RPMI-1640 media. COS-7 cells, a green monkey kidney cell line, were maintained in DMEM, and CHO-K1 (hereafter referred to as CHO) cells, a Chinese hamster ovary cell line, were grown in HAM F-12 nutrient mixture containing glutamine.

Labeling of Cellular Glycoproteins. Jurkat cells were seeded at a density of 2.0×10^5 cells/mL and incubated for 3 days in untreated media or media containing 35 μM Ac_4-ManNAz. After the incubation period, cells were collected, washed three times with PBS, resuspended in 25 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 2% Tween 20 (lysis buffer), and lysed through 10 freeze−thaw cycles. Remaining cellular debris was pelleted by centrifugation at 3000*g* for 30 min at 4 °C, and then the supernatant was subjected to Western blot or sialic acid analysis.

Expression of Recombinant Glycoproteins. The gene encoding IFN-β was amplified by PCR with the following primers: forward primer, 5′-CGAAGCTTCATGACCAA-CAAGTGTCTCC-3′; reverse primer, 5′-CCGAATTC-CGTTTCGGAGGTAACCTGTAAG-3′. The PCR product was digested with the restriction enzymes *Nhe*I and *Eco*RI and ligated into the vector pSecTag2A using T4 DNA ligase. The resulting plasmid encoded IFN-β with C-terminal Myc and hexahistidine tags (hereafter referred to as IFN-β). The plasmid encoding IFN-β was introduced into CHO cells using Lipofectamine Plus according to the manufacturer's instructions. GlyCAM-Ig (*38*) was introduced into CHO cells following a similar protocol except that plasmids encoding FucT7 and C2GnT were also added to generate elaborated glycans. Following the removal of the transfection media, OptiMEM or OptiMEM containing Ac_4ManNAz (35 μM) was added, and the cells were incubated for 4 d at 37 °C. The conditioned media were then collected and concentrated (typically 20-fold) using concentrators with a 10000 molecular weight cutoff. IFN-β was purified by metal affinity chromatography according to the manufacturer's instructions, except that Tween 20 (0.1%) was included in the buffers. Purified samples were analyzed by Western blot. For sialic acid analysis, IFN-β was purified further by gel excision of the bands at the appropriate molecular weight, essentially following the procedure described by Kuster et al. (*39*). The excised bands were washed twice with PBS for 30 min at room temperature and then once with acetonitrile:water (1:1) for 1 h at room temperature. The gel samples were cut into thirds, lyophilized, and then subjected to in-gel trypsin digestion (20 μg of trypsin/gel slice) in 20 mM ammonium bicarbonate buffer (pH 8.0, 200 μL) overnight at 37 °C. The resulting peptides were extracted from the gel by four cycles of sonication of the gel slices in acetonitrile:water (1:1, 600 μL) for 30 min at room temperature. The samples were lyophilized and subjected to acid hydrolysis as described below. GlyCAM-Ig was purified by incubation of the crude conditioned media with protein A−Sepharose beads. The beads were washed with PBS containing 0.05% Tween 20, and the protein was eluted with 100 mM glycine (pH 2.9). GlyCAM-Ig was determined to be >95% pure by Coomassie blue staining, and these samples were subjected to Western blot or sialic acid analysis.

Western Blot Analysis of Cellular and Recombinant Glycoproteins. For Western blot analysis of azide-labeled and unlabeled glycoproteins, all samples were separated by SDS−PAGE and transferred to nitrocellulose. Antibody labeling and subsequent detection with the SuperSignal West Pico chemiluminescent substrate was performed according to the manufacturer's instructions. For detection of azide-labeled glycoproteins, samples were preincubated with phosphine−FLAG (250 μM final concentration in PBS containing 4 M urea) overnight at room temperature prior to electrophoresis. Western blots of these samples were probed with anti-FLAG-HRP (1:3000 dilution). Western blots of IFN-β were probed with mouse anti-c-Myc (9E10) antibody followed by secondary detection with anti-mouse-HRP. GlyCAM-Ig was detected by probing with anti-IgG-HRP.

Sialidase Treatment of Glycoproteins. Lysates from Ac_4-ManNAz-treated cells were diluted into 50 mM sodium acetate (pH 5.5) and treated with sialidase from *V. cholerae* (2.2 milliunits, 20 μL total reaction volume) at 37 °C overnight. Subsequently, protein samples were reacted with phosphine−FLAG as described above and analyzed by Western blot.

HPLC and LC-MS Analyses of Sialic Acids. For sialic acid analysis, cell lysates and purified GlyCAM-Ig were acetone precipitated and resuspended in H_2O. These samples, or trypsin-digested IFN-β, were heated to 80 °C in 2 M acetic acid for 3 h. The released sialic acids were collected by ultrafiltration through a 3000 molecular weight cutoff filter and derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB) as described (*40*). The fluorescent, DMB-derivatized sialic acids were analyzed by reversed-phase HPLC using an Acclaim 120 C_{18} column (Dionex) at a flow rate of 0.9 mL/min. Samples were eluted with a gradient of acetonitrile (8−11%) in methanol (7%) and H_2O over 39 min followed by 11 min at the final conditions. The excitation and emission wavelengths were 373 and 448 nm, respectively. The DMB-derivatized sialic acids were identified and quantified by comparing elution times and peak areas to known standards that were similarly treated. The hydrolysates were analyzed by LC-MS using a similar reversed-phase HPLC separation except that the column was eluted isocratically with methanol (8%), acetonitrile (7%), formic acid (0.1%), and H_2O. The DMB-derivatized sialic acids were identified using both an on-line UV detector (373 nm) and a ThermoFinnagan LCQ mass spectrometer operated in the positive ion mode.

Monosaccharide Analysis. Cell lysates were treated with 2 M trifluoroacetic acid (TFA) at 100 °C for 4 h. Hydrolysates were dried, methanol was added, and the samples were dried again to ensure the complete removal of TFA. The samples were then analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a CarboPac PA-10 column (Dionex). Monosaccharides were eluted isocratically with sodium hydroxide (18 mM) at a flow rate of 1 mL/min. The identity of the sugars was determined by comparison of the elution times and peak areas to known standards that were similarly treated.

RESULTS

Azides Are Incorporated into Cellular Glycoproteins. With the goal of a general method for introducing bioorthogonal functional groups into sialylated glycoproteins, we first sought to define the scope of Ac_4ManNAz metabolism in cells. Although we had previously demonstrated that metabolism of Ac_4ManNAz produces azides within cell surface glycoconjugates, molecular characterization of the modified biomolecules had not been performed. There are several glycoconjugate subclasses, such as glycolipids and N- and O-linked glycoproteins. We first explored whether azides were resident in glycoproteins, a critical element for the proposed strategy.

We incubated three cell lines, Jurkat (T-cell lymphoma), Chinese hamster ovary (CHO), and COS-7 (Green monkey kidney epithelial) cells, with Ac_4ManNAz (35 μM) for 3 days. The cells were washed and lysed, and the cellular proteins were analyzed for azide labeling via Staudinger ligation with a phosphine reagent conjugated to the FLAG peptide (DYKDDDDK, phosphine−FLAG) (*25*). The labeled

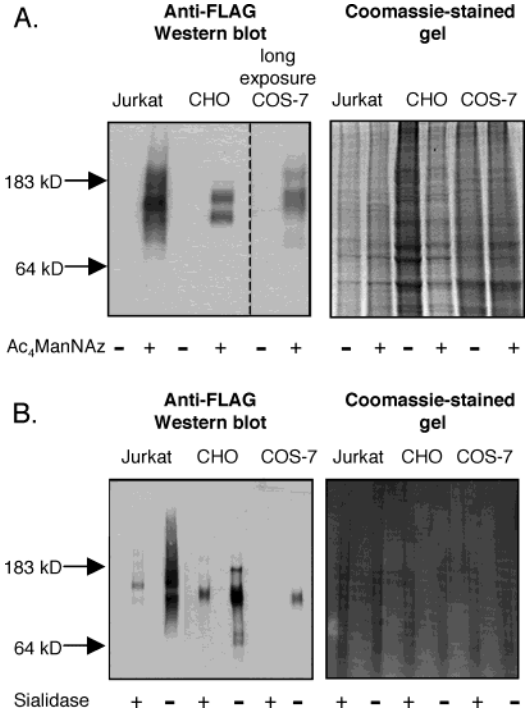

FIGURE 2: Western blot analyses of glycoproteins from cells treated with or without Ac_4ManNAz. (A) Jurkat, CHO, and COS-7 cells were untreated (−) or incubated with 35 μM Ac_4ManNAz (+) for 3 days, lysed, and labeled with phosphine−FLAG (250 μM). The samples were separated by SDS−PAGE and visualized by Coomassie staining (right) or transferred to nitrocellulose for detection with anti-FLAG-HRP (left). For samples from COS-7 cells, the exposure time of Western blot to film was 5-fold longer than for samples from Jurkat or CHO cells. (B) Glycoproteins from Ac_4ManNAz-treated Jurkat, CHO, and COS-7 cells were treated with *V. cholerae* sialidase (+) or untreated (−). The samples were reacted with phosphine−FLAG (250 μM) and visualized as in (A).

lysates were analyzed by Western blot probing with a peroxidase-conjugated anti-FLAG antibody (anti-FLAG-HRP) (*25*). As shown in Figure 2A, several glycoproteins from each cell type were found to be labeled with the FLAG peptide in a manner dependent on incubation of the cells with Ac_4ManNAz. Furthermore, lysates from Ac_4ManNAz-treated cells that were not exposed to phosphine−FLAG did not react with anti-FLAG-HRP (not shown). These data indicate that azides can be incorporated into glycoproteins expressed in diverse cell types. Labeling of proteins from Jurkat and CHO cells appeared to be more robust than from Ac_4ManNAz-treated COS-7 cells. These observations are consistent with previous flow cytometry data indicating that COS-7 cells present low levels of cell surface azides compared to the other cell types (*41*).

SiaNAz Is the Primary Metabolic Product of Ac_4ManNAz. On the basis of precedents with other unnatural mannosamines and on indirect evidence, we presumed that SiaNAz is the metabolic product of ManNAz (*28, 41*). However, this had not been directly demonstrated. Toward this end, the lysates were treated with a general sialidase (linkage-independent) that cleaves glycoconjugate-bound sialic acids from their underlying glycans. Although this enzyme had not previously been shown to recognize unnatural sialosides, sialidase treatment of lysates from Ac_4ManNAz-treated cells prior to phosphine−FLAG labeling significantly diminished the signal observed in Western blots

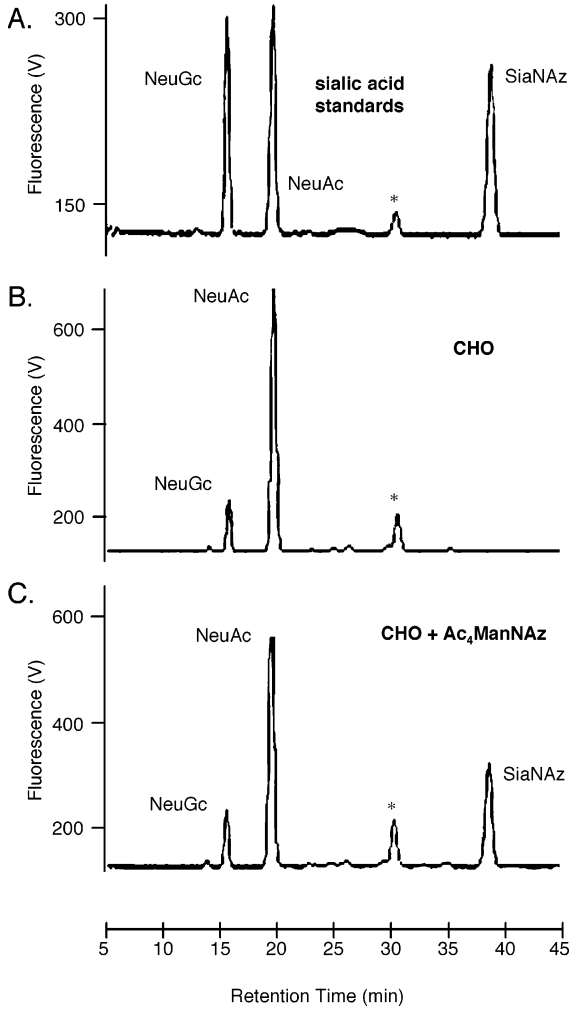

FIGURE 3: Representative HPLC traces of DMB-derivatized sugars from mild acid hydrolysis of CHO cell lysates. (A) Sialic acid standards indicating the retention times of NeuGc, NeuAc, and SiaNAz. (B) DMB-derivatized sialic acids released from lysates of untreated CHO cells. (C) DMB-derivatized sialic acids released from lysates of Ac_4ManNAz-treated CHO cells. The asterisk represents a contaminant observed in all samples.

(Figure 2B). The residual labeling observed after sialidase treatment may result from SiaNAz residues that are inaccessible to the enzyme. Alternatively, a minor fraction of the azides may reside within alternative metabolic products, the most likely of which is the glucosamine analogue GlcNAz (*41*, *42*).

To analyze SiaNAz directly, cell lysates were subjected to mild acid hydrolysis, a procedure known to selectively cleave sialic acid glycosides. The released sialic acids were isolated, derivatized with 1,2-diamino-4,5-methylenedioxybenzene (DMB), and analyzed by reversed-phase HPLC with fluorescence detection (*40*). The compounds identified by HPLC analysis were compared with natural monosaccharide standards and a synthetic sample of SiaNAz (*37*). Representative HPLC traces are shown in Figure 3B,C for CHO cell lysates obtained from untreated or Ac_4ManNAz-treated cells (trace A shows sialic acid standards). *N*-Acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) were present in both lysates. However, azide-labeled lysates contained a new peak with a retention time identical to that of DMB-derivatized SiaNAz. Similar elution profiles were obtained for released sialic acids derived from Jurkat

Table 1: Representation of Various Sialic Acids in Lysates from Jurkat, CHO, and COS-7 Cells[a]

cell line	Ac_4ManNAz	NeuGc	NeuAc	SiaNAz
Jurkat	−	3	97	0
Jurkat	+	3.5	56, 78	41, 18
CHO	−	16	84	0
CHO	+	12, 11	59, 69	29, 21
COS-7	−	8	92	0
COS-7	+	8, 7	87, 89	5, 4

[a] Cell lysates were subjected to mild acid hydrolysis. The resulting sialic acids were derivatized with DMB and analyzed by HPLC with fluorescence detection. The data are represented as the percentage of each individual sialic acid from the total of all sialic acid species detected. The results from duplicate measurements are reported for Ac_4ManNAz-treated samples.

and COS-7 cells, although the relative intensities of the peaks were different (not shown). By integration of the peaks from the HPLC profiles, the concentration of each released sialic acid (NeuAc, NeuGc, or SiaNAz) was obtained and converted to a value for percent of the total sialic acids (Table 1). Jurkat cells incorporated the most SiaNAz into their glycoproteins (41% of total sialic acid), followed by CHO (29%) and COS-7 cells (5%). These results are consistent with the Western blots from these samples. We noted that Jurkat cells that had been through many rounds of passaging incorporated SiaNAz at higher levels (41%) than cells that had been through fewer passages (18%).

Finally, we analyzed the acid hydrolysates of untreated and Ac_4ManNAz-treated cells by LC-MS. Compounds were observed with retention times and masses corresponding to those of the DMB-derivatized sialic acid standards, including SiaNAz (Figure 4). This is the first direct characterization of SiaNAz within cellular glycoconjugates.

Effects of Ac_4ManNAz on Global Sialoside Levels. Previous experiments from our laboratory have shown that unnatural sialic acids bearing extended alkyl side chains disrupt the elaboration of polysialic acid (PSA) in cells capable of producing this polymer (*43*). Although Jurkat, CHO, and COS-7 cells do not express the enzymes required for PSA biosynthesis, we sought to investigate whether monosialylation could be disrupted by interception of the sialic acid biosynthetic pathway with SiaNAz. To determine

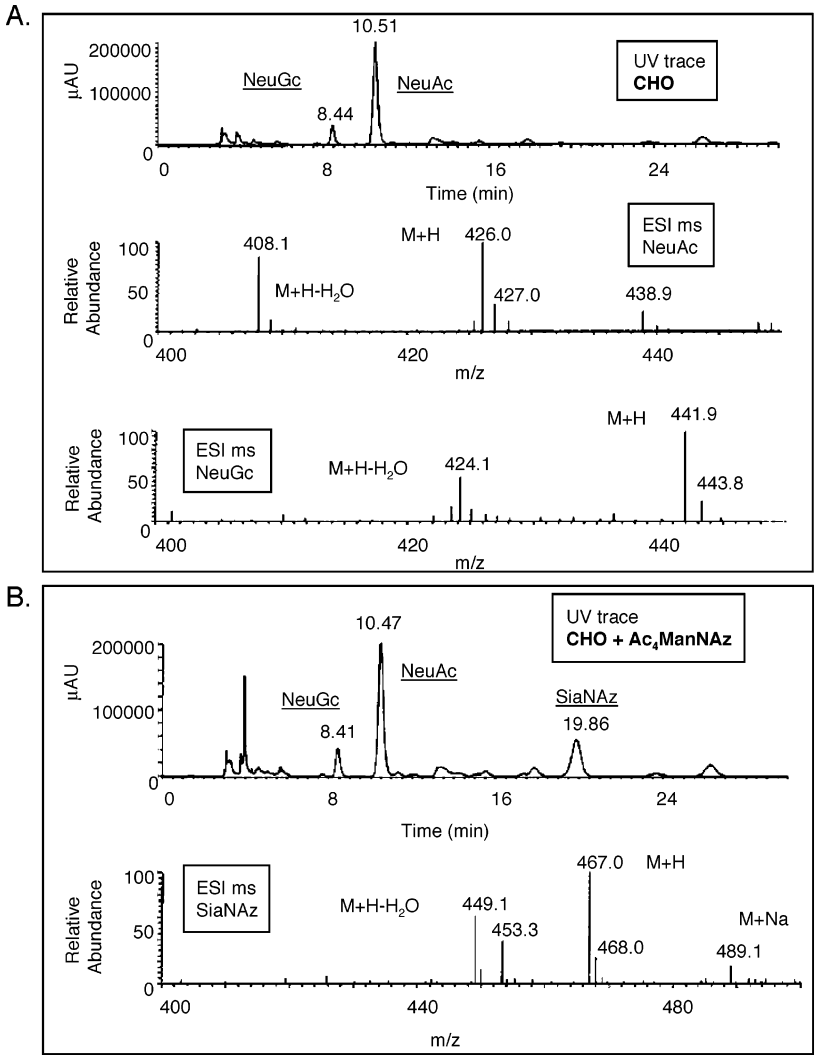

FIGURE 4: LC-MS analysis of DMB-derivatized sialic acids obtained following mild acid hydrolysis of CHO cell lysates. (A) HPLC trace with UV detection of samples from untreated CHO cells (top). Electrospray mass spectra of compounds with retention times at 10.51 (middle) and 8.44 (bottom) min revealed major ions for DMB-derivatized NeuAc and DMB-derivatized NeuGc, respectively. (B) HPLC trace with UV detection of samples from Ac_4ManNAz-treated CHO cells (top). Electrospray mass spectra of the compounds with retention times at 8.41 (not shown), 10.47 (not shown), or 19.86 (bottom) min indicated major ions for DMB-derivatized NeuGc, NeuAc, and SiaNAz, respectively.

Table 2: Sialic Acid:Monosaccharide Ratios from Lysates of Ac_4ManNAz-Treated (+) or Untreated (−) Jurkat, CHO, or COS-7 Cells[a]

cell line	Ac_4ManNAz	Sia:$GlcNH_2$	Sia:$GalNH_2$	Sia:Gal
Jurkat	−	0.7	2.6	2.0
Jurkat	+	0.6	2.2	1.5
CHO	−	0.7	2.1	1.2
CHO	+	0.9	3.0	1.6
COS-7	−	1.4	3.1	1.5
COS-7	+	0.6	1.5	0.9

[a] Lysates were subjected to strong acid hydrolysis. *N*-Acetylglucosamine and *N*-acetylgalactosamine were deacetylated to yield glucosamine and galactosamine, respectively, during acid hydrolysis.

whether CHO cells treated with Ac_4ManNAz were undersialylated, monosaccharide analysis was performed on lysates from cells that were untreated or treated with Ac_4ManNAz. The molar quantities of total sialic acids were compared with other monosaccharides found in the glycans. As shown in Table 2, the differences between the sialic acid:galactose ratios from untreated and Ac_4ManNAz-treated samples were within 2-fold for all samples tested. The sialic acid:glucosamine and sialic acid:galactosamine ratios for azide-labeled lysates were within 2.5-fold of the untreated samples. These data suggest that exposure of cells to Ac_4ManNAz does not grossly perturb sialylation of glycoproteins.

Expression of Azide-Labeled Recombinant Glycoproteins. Having confirmed that Ac_4ManNAz is converted to SiaNAz, which is then incorporated into cellular glycoproteins at significant levels in CHO cells, we next addressed whether recombinant secreted proteins could be labeled with the bioorthogonal functional group. Interferon-β (IFN-β) is an antiviral cytokine and immunomodulator that is currently used to treat multiple sclerosis (*2*, *44*). We chose IFN-β as the first target glycoprotein on the basis of the extensive biochemical characterization that has been performed on this protein and its therapeutic relevance. IFN-β possesses a single sialylated N-linked glycan at Asn80 that is known to be essential for protein stability (*45*, *46*). For ease of purification and detection, we engineered hexahistidine and c-Myc epitope tags onto the C-terminus of IFN-β. The corresponding plasmid was introduced into CHO cells by transient transfection, and the cells were incubated with or without Ac_4ManNAz for 4 days. IFN-β was purified from the conditioned media by metal affinity chromatography and probed for the presence of SiaNAz by phosphine−FLAG labeling and Western blot. IFN-β expressed in the presence of Ac_4ManNAz was immunoreactive with the anti-FLAG antibody at the expected molecular weight of the recombinant protein (Figure 5A). Purified IFN-β from untreated cells did not show immunoreactivity nor did azide-labeled IFN-β that was not treated with phosphine-FLAG. Furthermore, the immunoreactive species attributed to IFN-β was not present in the purified, conditioned medium from mock-transfected, Ac_4ManNAz-treated cells. Subsequent experiments demonstrated that azide labeling was dependent on the dose of Ac_4ManNAz, as shown previously for cell surface labeling (*41*), and sialidase treatment abrogated the azide-dependent signal (not shown). These data demonstrate that azides can be incorporated into the IFN-β glycan during recombinant overexpression and further derivatized by Staudinger ligation.

To determine if O-linked glycoproteins could also be expressed recombinantly with azide-labeled glycans, we next

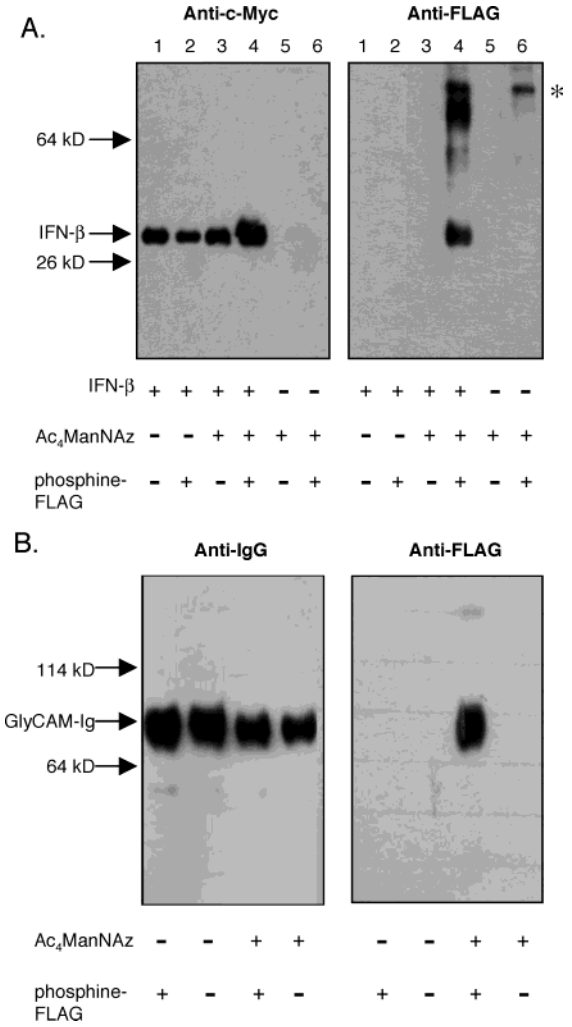

FIGURE 5: Recombinant glycoproteins expressed transiently in CHO cells in the presence (+) or absence (−) of Ac_4ManNAz. (A) IFN-β was collected from the conditioned media of transiently transfected cells treated with 35 μM Ac_4ManNAz (lanes 3 and 4) or untreated (lanes 1 and 2) and from Ac_4ManNAz-treated mock-transfected cells (lanes 5 and 6). Samples were purified by metal affinity chromatography, labeled with phosphine−FLAG (250 μM) where indicated (+), separated by SDS−PAGE, and analyzed by Western blot probing with mouse anti-c-Myc and anti-mouse IgG-HRP (left) or anti-FLAG-HRP (right). The high molecular weight bands observed in lanes 4 and 6 (indicated by the asterisk) appeared to be contaminating azide-labeled glycoproteins remaining after metal affinity chromatography. Some of these bands were also detected in the purified, conditioned media derived from mock-transfected cells. (B) GlyCAM-Ig was collected from the conditioned media of cells treated with 35 μM Ac_4ManNAz (+) or untreated (−). Samples were purified on protein A−Sepharose, reacted with phosphine−FLAG (250 μM) where indicated (+), separated by SDS−PAGE, and analyzed by Western blot probing with anti-IgG-HRP (left) or anti-FLAG-HRP (right).

examined the incorporation of SiaNAz into GlyCAM-1. This sialomucin is produced by lymph node high-endothelial venules and functions as a ligand for the leukocyte homing receptor L-selectin (*47*−*49*). The glycoprotein possesses ~40 potential sites of glycosylation dispersed in two clustered mucin domains. Recombinant soluble forms of GlyCAM-1 are of interest as L-selectin inhibitors for treatment of chronic inflammatory disease. GlyCAM-1 was expressed as a fusion protein with the Fc portion of a human IgG (GlyCAM-Ig) (*38*). The construct therefore has one N-linked glycan derived from the IgG heavy chain. CHO cells were transiently

Table 3: Identity of Glycoprotein-Bound Sialic Acids Released from Recombinant Glycoproteins Expressed from CHO Cells[a]

cell line	Ac_4ManNAz	NeuGc	NeuAc	SiaNAz
interferon-β	−	5	95	0
interferon-β	+	10, 6	62, 56	27, 38
GlyCAM-Ig	−	5	95	0
GlyCAM-Ig	+	7, 4	89, 85	4, 11

[a] Recombinant glycoproteins were subjected to mild acid hydrolysis. The resulting sialic acids were derivatized with DMB and analyzed by HPLC with fluorescence detection. The data are represented as in Table 1.

transfected with the plasmid encoding GlyCAM-Ig in the presence or absence of Ac_4ManNAz. The fusion protein was purified from the conditioned media on protein A−Sepharose and then labeled with phosphine−FLAG. Western blot analysis revealed robust labeling of GlyCAM-Ig from Ac_4-ManNAz-treated cells (Figure 5B). This labeling most likely reflects SiaNAz within O-linked glycans, as *N*-glycosidase F (PNGase F) treatment to enzymatically cleave the N-linked glycan from the fusion protein does not diminish labeling with the FLAG epitope (not shown). The identity of SiaNAz as the metabolic product of Ac_4ManNAz was confirmed by sialidase treatment (not shown) and sialic acid analysis (below).

We were interested in the relative levels of metabolic azide labeling of the two disparate glycoproteins. To quantitate these levels, the purified glycoprotein samples were desialylated by acid hydrolysis. The released sialic acids were derivatized with DMB and then analyzed by HPLC as above. SiaNAz constituted 38% of all sialic acids isolated from IFN-β (Table 3), similar to the SiaNAz levels observed in CHO cell lysates (Table 1). SiaNAz levels within GlyCAM-Ig were considerably lower, however, at 11% of total sialic acids (Table 3). These results suggest that different labeling efficiencies can be expected for different glycoproteins expressed in the same cells.

DISCUSSION

We have shown in this work that recombinant glycoproteins can be expressed with bioorthogonal functional groups resident in their glycans by metabolic oligosaccharide engineering. A series of experiments revealed that Ac_4-ManNAz is converted by cells to SiaNAz, which in turn is incorporated into cellular glycoproteins expressed in numerous cell lines (Jurkat, CHO, and COS-7). SiaNAz from cellular glycoproteins was directly identified by HPLC and LC-MS analyses of its DMB derivative. This is the first molecular level analysis of azido sugars incorporated into cellular glycans by metabolic means. The azides primed the glycoproteins for chemical modification with phosphine probes. It should be noted that azides have an alternative manifold of bioorthogonal reactivity, the [3 + 2] dipolar cycloaddition with alkynes (*50*). This provides an alternative means for derivatization of glycoproteins engineered with azido sugars.

As summarized in Tables 1 and 3, levels of SiaNAz labeling vary for different cell lines and for different glycoproteins from one cell line. There are several possible explanations for this observation. First, the cell lines may express different complements of sialyltransferases with various tolerances for the unnatural azide modification in their substrate, CMP−sialic acid. Second, different species orthologues of the enzymes that convert ManNAz to glycoconjugate-bound SiaNAz may differ in their unnatural substrate tolerances. Third, the cell lines may have different levels of endogenous ManNAc or other natural sialic acid pathway intermediates, thereby challenging the azido substrates with various levels of metabolic competition.

Notably, CHO cells, which are commonly used for the expression of recombinant glycoproteins on a large scale, replace 29% of natural sialic acids within their total glycoconjugate pool with SiaNAz. Since endogenous sialic acid biosynthesis is not suppressed, SiaNAz cannot replace all natural sialic acids even at high doses of Ac_4ManNAz. These data suggest that cell lines with lower levels of endogenous ManNAc will be superior hosts for metabolic glycoprotein labeling, and indeed, two such cell lines have recently been reported (*51*, *52*). Importantly, SiaNAz does not appear to grossly perturb overall sialylation (Table 2), indicating that near quantitative incorporation of SiaNAz into glycoproteins should be achievable in the ideal cell line.

We demonstrated that SiaNAz can be introduced into recombinant glycoproteins of therapeutic importance in both N- and O-linked glycans. The level of SiaNAz incorporation into IFN-β expressed in CHO cells was comparable to that observed in the total glycoprotein pool. By contrast, the incorporation efficiency in GlyCAM-Ig was lower than that of the total pool. It is possible that the sialyltransferases that act on the N-linked glycan of IFN-β and the O-linked glycans of GlyCAM-Ig have different tolerances for unnatural substrates. In this case, alterations in the expression levels or substrate recognition capabilities of limiting sialyltransferases may improve SiaNAz incorporation into recombinant glycoproteins. Alternatively, differences in the transit time of GlyCAM-Ig and IFN-β through the secretory pathway may affect their relative efficiencies of sialylation.

The ability to generate glycoproteins with bioorthogonal chemical handles provides a novel means for engineering modifications relevant to pharmaceutical development. The lifetime of glycoproteins in the serum can be dramatically altered by posttranslational and chemical modifications, and consequently, therapeutic agents are often modified to enhance their therapeutic efficacy (*14*). The introduction of a chemical handle into glycoproteins such as IFN-β will enable further derivatization for improvements to circulatory half-life, with molecules such as PEG or with ligands for tissue-specific receptors. This method of labeling is well suited for glycoproteins whose mechanism of action is determined by the protein component, since the modification site is distal from the polypeptide backbone and is unlikely to interfere with its function as a drug. For some glycoproteins such as EPO the extent of glycan sialylation has been correlated with increased serum half-life and in vivo efficacy (*10*, *11*). Unnatural modifications to sialic acid such as that demonstrated here may offer a method for resisting the activity of sialidases present in the serum, thereby extending serum half-life. Although the bacterial sialidase used in these studies was capable of cleaving SiaNAz residues, it is likely that further elaboration by Staudinger ligation would have blocked the activity of this enzyme and human sialidases. The same strategy could be applied to block interactions between sialylated carbohydrate ligands and their binding

partners in vivo as a means to improve our understanding of the role of glycoproteins in cell−cell communication.

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