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NMR Characterization of Immunoglobulin G Fc Glycan Motion on Enzymatic Sialylation

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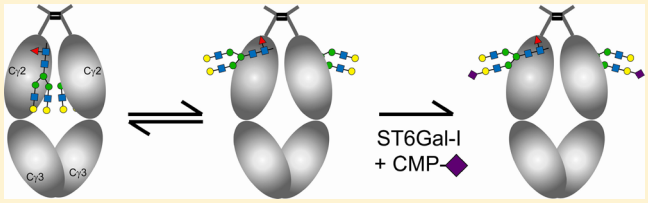
Supporting Information

ABSTRACT: The terminal carbohydrate residues of the *N*-glycan on the immunoglobulin G (IgG) fragment crystallizable (Fc) determine whether IgG activates pro- or anti-inflammatory receptors. The IgG Fc alone becomes potently anti-inflammatory upon addition of α2−6-linked *N*-acetylneuraminic acid residues to the *N*-glycan, stimulating interest in use of this entity in novel therapies for autoimmune disease [Kaneko et al. (2006) *Science 313*, 670−3]. Complete Fc sialylation has, however, been deemed challenging due to a combination of branch specificity and perceived protection by glycan−protein interactions. Here we report the preparation of high levels of disialylated Fc by using sufficient amounts of a highly active α2−6 sialyltransferase (ST6Gal1) preparation expressed in a transiently transformed human cell culture. Surprisingly, ST6Gal1 sialylated the two termini of the complex-type binantennary glycan in a manner remarkably similar to that observed for the free *N*-glycan, suggesting the Fc polypeptide does not greatly influence ST6Gal1 specificity. In addition, sialylation of either branch terminus does not appear to dramatically alter the motional behavior of the *N*-glycan as judged by solution NMR spectroscopy. Together these, data suggest the *N*-glycan occupies two distinct states: one with both glycan termini sequestered from enzymatic modification by an α1−6Man−branch interaction with the polypeptide surface and the other with both glycan termini exposed to the bulk solvent and free from glycan−polypeptide interactions. The results suggest new modes by which disialylated Fc can act as an anti-inflammatory effector.



Immunoglobulin G (IgG) is a remarkable molecule. Primarily thought of as a defense agent, IgGs are developed to adhere to an invading organism with high affinity and specificity. The fragment crystallizable (Fc) portion of engaged IgG is then recognized by cell surface receptors to initiate a pathogen destroying pro-inflammatory cascade.[1,2] However, Ravetch and co-workers recently reported that an uncommon glycoform, disialylated IgG, activates an anti-inflammatory receptor and has the potential therapeutic benefit of limiting or reversing damage from autoimmune disease.[3−5] The dramatic switch from pro-inflammatory to anti-inflammatory activity may involve direct receptor−glycan interactions or a more indirect effect on Fc structure, but the structural characterization of neither the disialylated glycan nor glycan-carrying Fc polypeptide has been reported. In the following we characterize critical properties of the glycan.

The engagement of IgG with cell surface receptors through the Fc portion of the molecule as shown in Figure 1 is largely independent of the antigen-binding Fab regions, but Fc−receptor engagement requires the presence of the conserved Fc *N*-glycan at Asn297.[6] The glycosylated Fc has thus become an accepted model for the study of IgG properties, and it is capable of potentiating a Fcγ Receptor (FcγR)-mediated pro-inflammatory response[7,8] or anti-inflammatory response.[4] Whether pro- or anti-inflammatory, the composition of the *N*-glycan termini strongly influences IgG Fc signaling. The *N*-glycan from human serum IgG is predominately a core-fucosylated complex type with two antennae (Figure 1b).[1] The most common glycoforms found on IgG Fc in human sera are those with either 0, 1, or 2 terminal galactose (Gal) residues; mono- and agalactosyl glycans are relatively more abundant in rheumatoid arthritis (RA) patients.[9] FcγRIIIa binds most strongly to Fc with an *N*-glycan truncated to the two α-linked mannose residues ($K_d \sim 2\ \mu M$)[7] and becomes weaker at each addition until the glycan termini are extended to the point where a sialylated Fc binds >300-fold more weakly ($K_d \sim 700\ \mu M$).[3] On the basis of these results, one might expect the receptor specifically recognizes the glycan; however, structures of the Fc−FcγRIII complex determined by X-ray diffraction clearly show the receptor bound to the Fc polypeptide in a position distal to the glycan termini and not contacting any part of the carbohydrate.[10,11] This observation is consistent with the IgG *N*-glycan termini playing a more complex structural role than simply as a binding epitope for an FcγR.

Modifying the glycan termini with *N*-acetylneuraminic acid, a sialic acid, reveals the anti-inflammatory properties of IgG.[3] *N*-Acetylneuraminic acid, often found on the termini of eukaryotic glycans, is essential for proper growth, development, and

Received: March 6, 2012
Revised: May 8, 2012
Published: May 10, 2012

dx.doi.org/10.1021/bi300319q | *Biochemistry* 2012, 51, 4618−4626

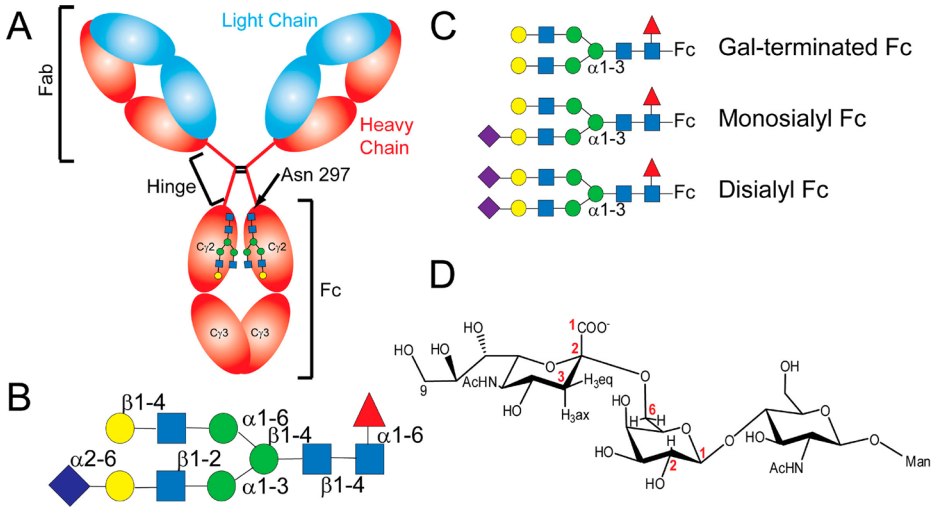


Figure 1. The IgG *N*-glycan, attached to Asn297 of the Fc heavy chain (A), is required for Fc-mediated signaling. (B) Though the composition of the Fc glycan in serum is heterogeneous, there is remarkably little variability in the types of inter-residue linkages observed. Note the mannose residues (green circles) are attached with either an α1−3 or α1−6 linkage which denotes the identity of each glycan branch built upon those residues. The symbols for the individual carbohydrate residues follow the Consortium for Functional Glycomics standard.[39] (C) Each of these three Fc glycoforms was studied here. "α1−3" refers to the α1−3Man-branch of the biantennary glycan as defined in the text. (D) Chemical structure of the glycan branch termini showing (from left to right) an *N*-acetylneuraminic acid α2−6 linked to a Gal residue β1−4 linked to an *N*-acetylglucosamine residue. Carbon positions enriched with ^{13}C nuclei are shown with red type. Glycans were enriched with either [$^{13}C_U$]- or [$^{13}C_2$]-Gal.

immune function.[12,13] Monosialylated IgG Fc represents <5% of all glycoforms, and disialylated Fc is rare if observed at all.[14] An IgG preparation, limited to sialylated forms by lectin chromatography, was shown to have unique anti-inflammatory properties in a mouse RA model.[4] The potency of this effect was increased >30−60-fold by enzymatic sialylation using the human β-D-galactoside α2−6 sialyltransferase, ST6Gal1, providing reasonable evidence that disialylated Fc was the active component of this mixture.[4] A possible receptor for disialylated Fc and a novel IL33-mediated anti-inflammatory signaling pathway were more recently identified.[5,15]

Obtaining large amounts of disialylated material to study the atomic details of anti-inflammatory Fc has proven challenging. Our previous work demonstrated that enzymatic sialylation of the α1−3Man-β1,2-GlcNAcβ1,4-Gal (here termed (α1−3Man-branch)-Gal) residue of a digalactose terminated Fc *N*-glycan was readily achieved, but sialylation of the α1−6Man-β1,2-GlcNAcβ1,4-Gal (here termed (α1−6Man-branch)-Gal) residue was very slow.[16] While this might have been expected based on the occlusion and surface interaction of the α1−6Man- branch seen in crystal structures,[17] ST6Gal1 was shown to sialylate the (α1−6Man-branch)-Gal residue more slowly than the (α1−3Man-branch)-Gal residue, even when using free *N*-glycan.[16,18] The Fc scaffold then further inhibits the rate of adding the first residue[16] and presumably further inhibits addition of the second residue. The sialylation conditions explored were, however, insufficient to measure both rates or generate substantial quantities of disialylated Fc.

An assessment of the Fc *N*-glycan dynamics by solution nuclear magnetic resonance (NMR) spectroscopy was also undertaken, but only for a construct with galactose termination on both branches.[19] These studies revealed that the glycan termini are quite mobile and experience rapid internal motions (τ_c 2−3 ns) in addition to the tumbling of the Fc dimer (τ_c ∼ 20 ns),[19] despite the ordered appearance of the carbohydrate in structures solved by X-ray crystallography.[17] Furthermore, the (α1−6Man-branch)-Gal residue experienced additional motion on a 100 μs time scale consistent with exchange of the α1−6Man-branch between a restricted protein-bound form and an uncoordinated highly dynamic form. These data suggest the *N*-glycan can be partially sequestered from the bulk solvent in a manner which may prevent contact with ST6Gal1 and various receptors. It is unclear, however, if the protein interaction affects only the α1−6Man-branch or both glycan branches and whether sialylation affects this interaction. Here we report the preparation of quantities of disialylated Fc, sufficient for structural and dynamic characterization, using high concentrations of highly active ST6Gal1. Measurements of sialylation kinetics and glycan dynamics lead to a two-state structural model in which both branches are exposed periodically on a 100 μs time scale, but with little perturbation by sialylation.

MATERIALS AND METHODS

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Expression, Purification, and Assay of GFP-ST6Gal1. The catalytic domain of rat ST6Gal1 was expressed as a soluble, secreted fusion protein by transient transfection of HEK293 suspension cultures. The coding region was comprised of a 25 amino acid NH_2-terminal signal sequence from the *T. cruzi* lysosomal α-mannosidase[20] followed by an 8xHis tag, 17 amino acid AviTag,[21] "superfolder" GFP,[22] the 7 amino acid recognition sequence of the tobacco etch virus (TEV) protease, and residues 95−403 of rat ST6Gal1 (Uniprot P13721). The entire coding region was codon optimized for human cells, synthesized by GeneArt AG (Regensburg, Germany), and subcloned using *Not*I and *Hin*dIII restriction sites into a chemically synthesized vector containing promoter, intron, postregulatory element, termination, and terminal repeat sequences similar to the pXLG vector[23] within the backbone of the bacteria shuttle plasmid, pMA (GeneArt). This expression vector was designated ST6Gal1-pGEn2, and the recombinant product was termed GFP-ST6Gal1.

dx.doi.org/10.1021/bi300319q | *Biochemistry* 2012, 51, 4618−4626

Suspension culture HEK293f cells (Life Technologies, Grand Island, NY) were grown to 1.6×10^6 cells/mL in Freestyle 293 Expression Medium (Life Technologies) at 37 °C and transfected with the ST6Gal1-pGEn2 expression vector using the Transit-Pro transfection reagent including Pro Boost Reagent in OptiPro SFM (Life Technologies) medium as described by the manufacturer (Mirus, Madison, WI). The culture was harvested 6 days following transfection, and the medium was clarified by centrifugation at 3500 rpm for 5 min. The medium was adjusted to contain 20 mM imidazole and loaded onto a column containing 25 mL Ni-NTA superflow (Qiagen, Valencia, CA) equilibrated with 20 mM HEPES, 300 mM NaCl, 20 mM imidazole, pH 7.2 (buffer A). Following the loading of the sample, the column was washed with 150 mL of buffer A and eluted first with 60 mL of buffer A containing 50 mM imidazole, followed by 60 mL of buffer A containing 100 mM imidazole and 250 mL of buffer A containing 300 mM imidazole. Fractions containing fluorescence (300 mM imidazole elution) were pooled and concentrated to ~2.5 mL using an ultrafiltration pressure cell membrane (Millipore, Billerica, MA) with a 10 kDa molecular weight cutoff. The concentrated sample was then loaded onto a Superdex 75 column (GE Healthcare Life Sciences, Pittsburgh, PA) preconditioned with a buffer containing 20 mM HEPES, 300 mM NaCl, 60 mM imidazole, pH 7.4. Fractions containing fluorescence were concentrated by ultrafiltration. A 200 mL initial culture yielded 12 mg of purified protein at a final concentration of 2.9 mg/mL.

Enzyme activity measurements for the rat ST6Gal1-GFP fusion protein and a commercial preparation of human α2,6-(*N*)-sialyltransferase (Calbiochem) were obtained through the use of a phosphatase-coupled assay utilizing colorimetric detection of inorganic phosphate with malachite green-based reagents.[24] Reactions were carried out for 1 h at 37 °C in the presence of 1.5 mM CMP-*N*-acetylneuraminic acid as a sugar donor and 4 mM lactosamine as glycan acceptor. Assay reactions contained 0.03 μg of recombinant human CD73 phosphatase in a buffer of 50 mM Bis-Tris, pH 6.5, and ST6Gal1 (0.05−0.5 μg per reaction) in a final volume of 20 μL. CMP generated through ST6Gal1 action was hydrolyzed by CD73 to release free phosphate. After incubation, reaction aliquots were diluted either 1:4 or 1:24 to expand the dynamic range of the assay, and reactions were developed using the malachite green reagent (R&D Systems). Absorbency was measured at 620 nm as described,[24] and released inorganic phosphate was determined using a phosphate standard curve and converted to units of μmoles of product generated min^{-1} mg^{-1} protein.

Preparation of Mono- and Disialylated Fc. Fc, highly enriched (~90%) in the monosialylated glycoform, was prepared as described previously[16,19] using 100 mU/mL α2−6 sialyltransferase purchased from Calbiochem (Sand Diego, CA). For kinetic measurements of sialylation, Fc was sialylated in a buffer containing 25 mM MOPS, 100 mM potassium chloride, 1 mM CMP-*N*-acetylneuraminic acid, 5 mg/mL Fc, and 0.05−0.5 mg/mL GFP-ST6Gal1 prepared as described in the previous section, pH 7.2, and incubated at 37 °C. The extent of sialylation was monitored by MALDI-MS as described previously[16] using an Applied Biosystems SCIEX TOF/TOF 5800 mass spectrometer (Carlsbad, CA).

For NMR experiments, Fc with a high proportion of disialyl-terminated *N*-glycans was prepared by sialylating in a buffer containing 25 mM MOPS, 100 mM potassium chloride, 1 mM CMP-[$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid (prepared as described[25]), 15 mg/mL Fc (Athens Research and Technologies, Athens GA), and 2.5 mg/mL GFP-ST6Gal1, pH 7.2. This reaction was incubated at 37 °C for 24 h. Following incubation the sample was buffer exchanged with 25 mM MOPS, 100 mM potassium chloride, pH 7.2, using an Amicon 10 kDa cutoff spin concentrator (Millipore, Billerica, MA) to remove CMP, an inhibitor of ST6Gal1 activity.[26] Next, CMP-[$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid was added back to this washed preparation before another 24 h incubation. This incubation/buffer exchange process was repeated twice, followed by a final fourth 24 h incubation at 37 °C, and resulted in a highly disialylated Fc preparation (>75%).

Kinetic Analysis of Sialylation. The accumulation of Fc glycoforms was monitored as described above and fit to a simple model for the sequential addition of *N*-acetylneuraminic acid to each of two Gal termini on a complex-type biantennary glycan (*A*) as shown in Figure 1c:

$$E + A \Leftrightarrow EA \rightarrow E + B \Leftrightarrow EB \rightarrow E + C \quad (1)$$

Here E is GFP-ST6Gal1, A is defined above, B is monosialyl Fc, C is disialyl Fc, and EA and EB are Michaelis complexes; CMP-*N*-acetylneuraminic acid was assumed to be in excess for the entirety of the reaction, and CMP concentrations were well below inhibitory levels. Rate equations for the accumulation of A, B and C were obtained using the King−Altman method[27] on the BioKin Web site. Rate equations were numerically integrated, resulting in the accumulation curves shown. The catalytic steps were assumed to be the slowest processes in this mechanism, and differences in the rate of the first and second catalytic steps were fit directly.

NMR Spectroscopy. Fc enriched with ([$^{13}C_2$] or [$^{13}C_U$]) galactose and [$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid was prepared with either one *N*-acetylneuraminic acid primarily on the α1−3Man-branch or both branches terminated with an *N*-acetylneuraminic acid residue as described above. Glycan-remodeled Fc (5 mg) was exchanged into a buffer containing 25 mM sodium phosphate, 100 mM potassium chloride, 1 mM DSS in 100% D_2O, pH 7.0, using an Amicon 10 kDa cutoff spin concentrator (Millipore, Billerica, MA) and adjusted to a final volume of ~200 μL.

NMR experiments were performed on a spectrometer operating at 21.1T and equipped with a Varian VNMRS console as well as a cryogenically cooled 5 mm probe (Agilent Technologies, Santa Clara, CA). All NMR experiments were performed at 50 °C unless otherwise noted. Pulse sequences for measuring R_1, $R_{1\rho}$ (using a continuous-wave ^{13}C spin-lock[28]), and R_2 (using a Carr−Purcell pulse sequence element[29]) relaxation rates are shown in Figure S1. The one-dimensional spectra were the result of either direct observation of ^{13}C magnetization (S1a) or indirect detection of ^{13}C through ^{1}H magnetization (S1b,c). The latter proved to be of superior sensitivity. The rate of signal intensity decrease was fit with an equation describing a simple-exponential decay.

Gal resonances in the NMR spectra were assigned based upon similarities to those observed previously.[19] The *N*-acetylneuraminic acid resonances were assigned according to the order of their appearance and the branch specificity of ST6Gal1.[16]

RESULTS

Enzymatic Sialylation of Fc. Attempts to prepare milligram quantities of disialylated Fc, from Fc decorated

with a digalactosyl-terminated glycan, using accepted protocols and amounts of ST6Gal1 readily available from commercial sources proved difficult despite repeated attempts.[4,16] At best, the ratio of monosialylated to disialylated Fc was ~15:1 following sialylation, purification, and resialylation as shown in a MALDI-MS spectrum of the released glycan in Figure 2a.

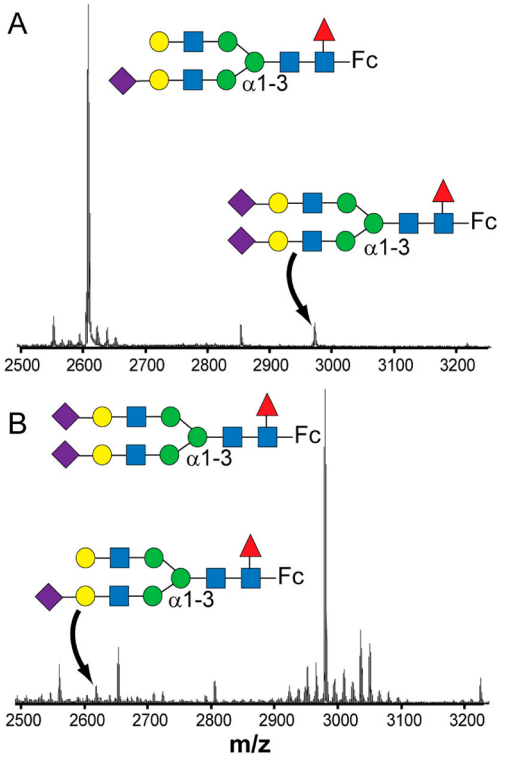


Figure 2. MALDI-MS spectrum of the liberated and permethylated *N*-glycan following Fc sialylation using GFP-ST6Gal1. Primarily monosialylated (A) or disialylated (B) material was prepared depending on the amount of enzyme used and the length of incubation.

Subsequent MS and NMR analysis showed sialylation to be nearly exclusively on the α1−3Man-branch.[16] Thus, we pursued a recently developed protocol to express rat ST6Gal1 and to test sialylation at greater enzyme concentrations. A truncated form of the rat ST6Gal1 coding region, devoid of its N-terminal transmembrane domain sequence, was assembled as a C-terminal fusion to an N-terminal signal sequence, 8xHis tag, AviTag, GFP, and TEV protease recognition sequence in a custom mammalian expression vector driven by the CVM promoter (ST6Gal1-pGEn2). The construct was transiently transfected into HEK293 suspension cultures grown in serum-free medium, and the recombinant product was purified from the conditioned medium by Ni^{2+}-NTA chromatography and gel filtration. Following the final purification step, the recombinant protein (GFP-ST6Gal1) was of high purity, shown in Figure 3, and as active as a commercial preparation of human ST6Gal1 (2.2 ± 1.0 vs 2.5 ± 0.5 μmol min^{-1} mg^{-1}, respectively). The yield was ~60 mg of purified GFP-ST6Gal1 per liter of culture medium. In a separate experiment, GFP-ST6Gal1 was expressed in dropout medium containing ^{15}N-Phe as the amino acid source to isotopically label the product. After cleaving the fusion construct with TEV protease and separating the ST6Gal1 portion by Ni^{2+}-NTA chromatography, an ^{15}N-heteronuclear single quantum coherence (HSQC) experiment

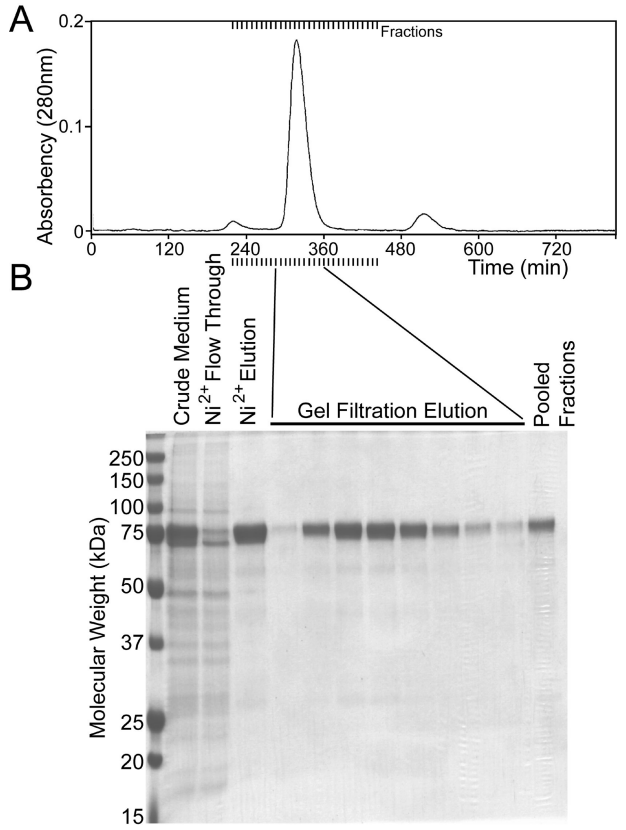


Figure 3. Purification of GFP-ST6Gal1 from transiently transformed human HEK293F cells. (A) Gel filtration chromatograph of GFP-ST6Gal1 followed initial purification by immobilized metal ion (Ni^{2+}) chromatography. (B) SDS-PAGE gel of the purification steps and final purified material.

was observed to be virtually identical to spectra taken of ST6Gal1 expressed with stably transformed HEK293 cells in adherent cultures[30] [Lee, H.-W., Moremen, K. W., and Prestegard, J. H., unpublished data].

An initial attempt to prepare disialylated material with upward of 0.4 mg of GFP-ST6Gal1 and 5 mg of desialylated IgG-Fc per mL of reaction was successful and showed nearly complete Fc sialylation (Figure 2b). A pair of time-course reactions, with 0.05−0.5 mg/mL GFP-ST6Gal1, revealed the accumulation of disialylated Fc and the transient nature of the monosialylated intermediate, as shown in Figure 4. Given our previous observation of initial sialylation on the α1−3Man-branch, we fit the kinetic data with a simple stepwise addition model as shown in the scheme at the bottom of Figure 4. This revealed the formation of monosialyl Fc with a rate 11-fold greater than the formation of disialylated Fc. The enhanced rate for sialyation on the α(1−3)Man-branch is also consistent with previous reports on sialylation of complex-type biantennary glycans by ST6Gal1 from other species.[16,18,31]

NMR Analysis of the Sialylated Fc. Larger quantities (>5 mg) of mono- and disialylated Fc were prepared to investigate the glycan structure by solution NMR spectroscopy. A region showing the Gal resonances in a two-dimensional ^{13}C-HSQC spectrum of Fc labeled with [$^{13}C_U$]Gal and monosialylated on the α1−3Man-branch showed many similarities when compared to a similar spectrum of [$^{13}C_U$]-Gal-terminated Fc without sialylation (Figure 5a,b). One notable similarity was the relatively greater intensity of the (α1−3Man-branch)-Gal

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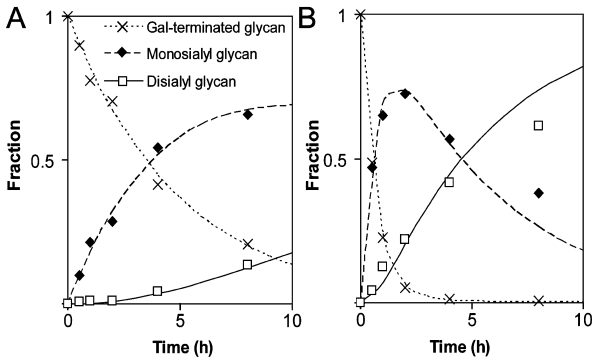

Figure 4. Plots of the accumulation of monosialyl and disialyl Fc in the presence of GFP-ST6Gal1 and CMP-*N*-acetylneuraminic acid were fitted to reveal the relative rates of sialylation on each glycan branch terminus. The reaction with 0.05 mg/mL (A) or 0.5 mg/mL (B) GFP-ST6Gal1 was monitored by MALDI-MS. By fitting these data to an equation that describes the accumulation of product according to a sequential addition model, the rate of the addition of the first residue was found to be ~11-fold faster than the rate of the second residue. This result is similar to the 9-fold difference observed when reanalyzing similar data for the sialylation of free *N*-glycan using bovine ST6Gal1[18] or 8-fold for human ST6Gal1.[16]

resonances when compared to those on the α1−6Man-branch. Likewise, the positions of the (α1−6Man-branch)-Gal resonances were largely unchanged except those for the C6 linkage site and the adjacent C5 carbon. The latter are expected effects of linkage through the Gal C6 to an *N*-acetylneuraminic acid. These data are consistent with a monosialylated Fc *N*-glycan that is similar in conformation and dynamics to that of the Gal-terminated *N*-glycan described previously.[19]

A ^{13}C-HSQC of disialylated Fc containing [$^{13}C_U$]-Gal also shows the line widths and relative positions of the Gal peaks to be largely similar to those of the (α1−6Man-branch)-Gal resonance in the monosialylated and sialyl-free Fc spectra (Figure 5c). Interestingly, however, the (α1−6Man-branch)-Gal H2−C2 cross-peak in the disialylated form (75.48 ppm) is shifted upfield in the carbon dimension as compared to its position in a spectrum of monosialylated Fc (75.87 ppm); this is unlike the behavior of the (α1−3Man-branch)-Gal H2−C2 cross-peaks on comparing the monosialylated and sialyl-free forms (Figures 5b and 5a) which show little change in positions. The Gal-C2 peak shows a large difference between α1−3Man-branch and α1−6Man-branch in both Gal and *N*-acetylneuraminic acid terminated glycans, something that was deemed to originate in glycan−protein contacts for the Gal terminated Fc.[19]

Spectra of [$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid resonances on Fc indicate the behavior of both (α1−3Man-branch)- and (α1−6Man-branch)-*N*-acetylneuraminic acid residues is quite similar (Figure 5d,e). As expected, the α1−3Man-branch resonances on both mono- and disialylated Fc are intense and show line widths comparable to those of Gal residues on the same branch. Surprisingly, however, the H3−C3 resonances of (α1−6Man-branch)-*N*-acetylneuraminic acid were also narrow and intense. This characteristic is in contrast to the broad peaks seen for the underlying (α1−6Man-branch)-Gal resonances. Despite similar peak characteristics the (α1−3Man-branch)- and (α1−6Man-branch)-*N*-acetylneuraminic acid cross-peaks do show chemical shift differences (^{13}C3 chemical shifts = 42.93 and 42.61 ppm, respectively), suggesting differences in chemical environments similar to those observed for most of the Gal carbons.

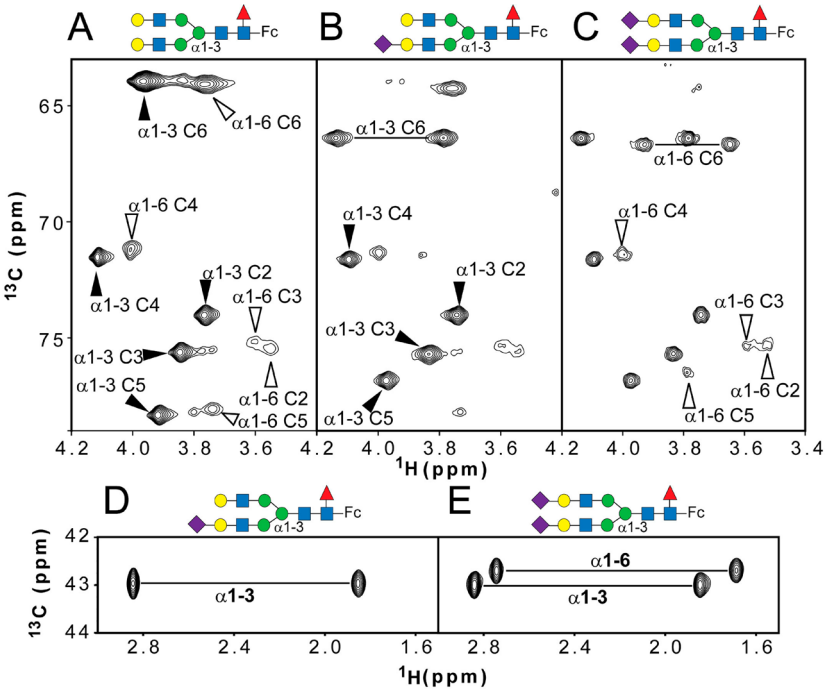

Figure 5. NMR spectroscopy of terminal Gal and *N*-acetylneuraminic acid residues of the Fc-conjugated *N*-glycan shows distinct ^{1}H−^{13}C correlations. (A) [$^{13}C_U$]-Gal resonances observed in a ^{13}C-HSQC spectrum of Gal-terminated Fc. (B) A similar experiment using Fc with a [$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid residue attached to the (α1−3Man-branch)-Gal residue. (C) ^{13}C-HSQC spectrum of di[$^{13}C_U$]-Gal di[$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid Fc. (D) and (E) ^{13}C-HSQC spectrum of the $^{13}C_3$ *N*-acetylneuraminic acid region for the material in (B) and (C), respectively. "α1−3" and "α1−6" refer to the Man residue at the base of the glycan branch to which the residue is attached.

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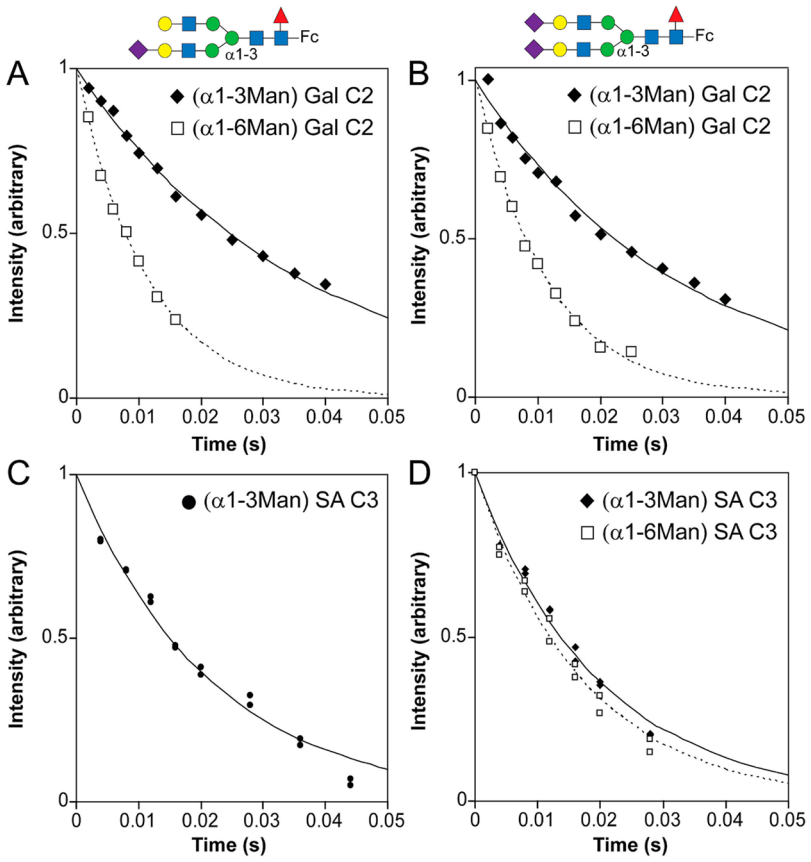

Figure 6. The two glycan branch termini behave differently according to NMR relaxation measurements of the Fc-conjugated *N*-glycan. The decay of signal intensity in Carr−Purcell R_2 relaxation experiments specific for [$^{13}C_2$]-Gal (A, B) and [$^{13}C_3$]-*N*-acetylneuraminic acid (C, D) nuclei is shown. Measurements were performed with both monosialyl-Fc (A, C) and disialyl Fc (B, D). The effect of $^{13}C_3$−$^{13}C_2$ coupling during the $^{13}C_3$ *N*-acetylneuraminic acid measurements was removed by inverting the $^{13}C_2$ spin with a selective pulse coincident with ^{1}H decoupling. "SA" is shorthand notation for *N*-acetylneuraminic acid.

Dynamics of the Sialylated Fc *N*-Glycans. Measurements of Gal resonance relaxation rates for these sialoforms were performed and are shown in Figure 6 and Table 1. The relaxation rates of the Gal resonances for the mono- and disialyl Fc were largely similar to those measured for the Gal-terminated Fc. This suggests the Gal termini experience similar motion on the fast nanosecond time scale and similar slow microsecond time scale dynamics as observed with the Gal-terminated Fc previously.[19] It does appear as though the (α1−6Man-branch)-Gal residues experience slightly less motion when one or both *N*-acetylneuraminic acids are present based upon the slightly smaller R_1 values and greater $R_{1\rho}$ and R_2 values.

In contrast to relaxation of the Gal ^{13}C2 nuclei, C3 nuclei from both *N*-acetylneuraminic acid residues in the disialyl sialoform had similar relaxation rates. This observation is consistent with the line shape and intensity similarity seen in Figure 5e. The R_1 values for the *N*-acetylneuraminic acid C3 nuclei on both branches are about twice that of the C2-Gal on the α1−3Man-branch. However, in the limit of uncorrelated spin−spin interactions and isotropic motion with the same correlation time as the C2-Gal, one expects a factor of 2 increase because of the two protons on the *N*-acetylneuraminic acid C3 carbon. R_2 and $R_{1\rho}$ are also close to, or less than, a factor of 2 larger, suggesting fast internal motions similar to those experienced by the Gal residue of the α1−3Man-branch.

Temperature Dependence of Gal Chemical Shifts. Assessing spectral properties across a range of temperatures can provide insight into both dynamic processes and population changes of two states. Previously, temperature-induced changes of the (α1−6Man-branch)-[$^{13}C_2$]-Gal resonance position were suggested to be correlated with populations of two α1−6Man-branch states: one with well-defined interactions with the protein surface and one being a dynamic average of an ensemble of less restrained conformations, the chemical shifts of which are identical to a solvent-exposed glycan.[19] The (α1−6Man-branch)-[$^{13}C_2$]-Gal resonance moved from a chemical shift of 75.3 ppm at the high temperature limit to 76.4 ppm at the low temperature limit. At a temperature of 50 °C the glycan exchanged rapidly (~5000 s^{-1}) between these two states, giving rise to an average chemical shift. Thus, the proportion of the glycan in each state could be estimated from the chemical shift position. A chemical shift value <75.85 ppm indicates the glycan is predominately in the highly dynamic state, and a value >75.85 ppm indicates a dominance of the more restricted state.[19] The chemical shift of the (α1−6Man-branch)-[$^{13}C_2$]-Gal resonance on the monosialylated Fc at 25 °C is slightly greater (75.87 ppm) than that observed for the Gal-terminated Fc (75.69 ppm), suggesting the motion of this glycan branch is more restricted on the monosialyl Fc. The corresponding resonance of disialyl Fc has a slightly lesser value (75.48 ppm) at 25 °C and, assuming no direct chemical shift perturbations

dx.doi.org/10.1021/bi300319q | *Biochemistry* 2012, 51, 4618−4626

Table 1. Relaxation Parameters for the Fc-Conjugated Asn297 *N*-Glycan

nucleus	parameter	Gal-terminated[a] (s^{-1})	monosialyl Fc (s^{-1})	disialyl Fc (s^{-1})
α1−3 Gal C2	R_1	1.2 ± 0.1	1.31 ± 0.04	1.9 ± 0.1
	$R_{1\rho}$	20 ± 1	27 ± 1	29 ± 1
	R_2 CP	n.d.	28 ± 1	31 ± 2
	R_2 line width	40	43	41
α1−6 Gal C2	R_1	1.2 ± 0.2	1.1 ± 0.1	0.9 ± 0.1
	$R_{1\rho}$	56 ± 10	68 ± 5	75 ± 4
	R_2 CP	n.d.	90 ± 3	88 ± 3
	R_2 line width	∼150	∼180	∼170
α1−3 SA C3	R_1	n.a.	3.6 ± 0.2	3.8 ± 0.3
	$R_{1\rho}$	n.a.	26.7 ± 0.9	26 ± 4
	R_2 CP	n.a.	46 ± 3	51 ± 3
	R_2 line width	n.a.	51	53
α1−6 SA C3	R_1	n.a.	n.a.	4.2 ± 0.2
	$R_{1\rho}$	n.a.	n.a.	36 ± 6
	R_2 CP	n.a.	n.a.	59 ± 4
	R_2 line width	n.a.	n.a.	53

[a]Barb and Prestegard (2011) *Nat. Chem. Biol.* 7, 157−153.

due to sialylation of the (α1−6Man-branch), would be less restricted.

DISCUSSION

In summary, the availability of a highly active form of the sialyltransferase, ST6Gal1, in large quantities has allowed preparation of a rare and potentially biomedically important glycoform of IgG-Fc. IgG Fc is a unique glycoprotein with described roles as a mediator of both pro- and anti-inflammatory mechanisms.[4,32,33] However, previous attempts had failed to produce high levels of disialylated Fc, suggesting the presence of the Fc polypeptide inhibited complete sialylation.[16] To overcome this inhibition large amounts of enzyme, produced in a cost-effective manner by way of an optimized transient human cell expression system, were utilized. Both nearly homogeneous mono-(α1−3Man-branch)-*N*-acetylneuraminic acid and disialylated Fc fractions have been prepared. This illustrates the value of an improved production system for glycan modifying enzymes and their use in remodeling glycoproteins to those with enhanced therapeutic value. The availability of the sialylated versions of IgG Fc specifically allows the characterization of the glycans as a basis for understanding their functional effects.

Glycan Motion and Accessibilty. Both the sialylation kinetics and glycan dynamics provide clues to the nature of large-scale Fc glycan motions. The rate of Fc *N*-glycan sialylation is slower than that of a free glycan, indicating the Fc polypeptide does inhibit sialylation,[16] but only by a factor of 5 or so. Comparing the preference of rat ST6Gal1 for (α1−3Man-branch)-Gal sialylation over (α1−6Man-branch)-Gal sialylation on unattached glycan to its preference on Fc-conjugated glycans reveals a striking similarity (Figure 4). Reanalysis of the kinetic data reported by Joziasse et al.[18] on the bovine ST6Gal1-catalyzed sialylation of the free biantennary complex-type glycan according to our simple kinetic model revealed a difference in branch sialylation preference of 9-fold (data not shown). A similar result showing a 7-fold difference was obtained by fitting our previous report[16] of human ST6Gal1 sialylation of a free Fc *N*-glycan (data not shown). These differences are remarkably similar to the 11-fold difference we report here and suggest the specificity of ST6Gal1 is unchanged by the presence of the Fc polypeptide. It is important to note that these different forms of the enzyme are highly similar (>92% identical or strongly similar at the amino acid level).

The NMR relaxation measurements are consistent with largely similar glycan dynamic profiles across the three sialoforms. Only minor differences in resonance frequencies or relaxation rates for Gal residues were observed. It is somewhat surprising that remodeling the glycan termini has such a small effect on the Gal residue and thus branch behavior. The narrow line widths and parallel changes in relaxation rates for *N*-acetylneuraminic acid residues on both branches suggest that these residues are highly dynamic and free of strong interaction with Fc polypeptide residues regardless of their branch identity. Thus, the glycan−polypeptide interactions are dominated by carbohydrate residues up to and including Gal, but not the *N*-acetylneuraminic acids. It is important to note that the measurements reported here are not sensitive to some time scales of motion, notably those between 20 ns and ∼100 μs.

The above-described glycan accessibility and NMR dynamics observations are consistent with a pair of Fc glycan structures that rapidly interconvert in solution (on the order of 5000 s^{-1}).[19] In one of these, the *N*-glycan is characterized by both glycan termini occupying conformations that are exposed to bulk solvent and therefore accessible to glycan modifying enzymes in much the same way they are in a released and isolated glycan. This is the dominant form at 50 °C. The second state has both *N*-glycan termini sequestered from the bulk solvent by an interaction with the polypeptide surface mediated by the α1−6Man-branch. This state dominates at lower temperatures. These models are consistent with numerous reports of enzymatic glycan remodeling (references including, but not limited to, refs 16, 34−36). Molecular dynamics simulations may hold the key to understanding the glycan transition between exposed and sequestered states as well as provide an indication of conformations accessible to the exposed glycan.

An Interpretation of the Anti-Inflammatory Properties of Disialyl Fc. The anti-inflammatory role of Fc has been attributed to the disialyl Fc fraction of a sialylated Fc preparation.[3,4] It is thus of interest to explore the possible molecular determinants behind this switch of Fc from a pro- to anti-inflammatory molecule upon glycan sialylation. This could be easily explained if dendritic-cell specific ICAM3-grabbing nonintegrin (DC-SIGN), the anti-inflammatory receptor suggested for disialyl Fc,[5,15] showed affinity for *N*-acetylneuraminic acid-terminated glycans. However, DC-SIGN has failed to bind sialylated complex-type glycans in multiple glycan arrays (refs 37, 38 and CFG core-H experiment 2235 www.funtionalglycomics.org). Thus, alternative hypotheses must be explored.

The data presented here show a high degree of similarity between the *N*-glycan dynamics and structural profiles for these three Fc sialoforms (Figure 5, 6 and Table 1), making it unlikely that there dramatic changes in protein conformation or

dx.doi.org/10.1021/bi300319q | *Biochemistry* 2012, 51, 4618−4626

polypeptide exposure result from direct interactions with sialic acids. However, our analysis of chemical shift changes of the C2 Gal residue of the α1−3Man-branch do suggest a small shift to the more exposed state on disialylation of the glycan. This could either allow enhanced glycan accessibility or a subtle change in Fc polypeptide conformation due to a shift away from the state having interactions with the α1−6Man-branch. Of course, the exclusion of such subtle, but important, changes will have to await detailed examination of the structure of the polypeptide portion of the various Fc glycoforms. Given the exposure of the glycan in one of the two equilibrating forms suggested by our data, it is possible that the *N*-acetylneuraminic acids bind more remote parts of receptors like DC-SIGN and may even a partially expose a core fucose to facilitate DC-SIGN interactions. It is also possible that the differences between pro- and anti-inflammatory Fc can be very subtle. A slightly higher affinity for an anti-inflammatory receptor at the head of an anti-inflammatory cascade could in principle lead to a lower threshold for activation of a more powerful anti-inflammatory response. Further experiments will be required to distinguish these possibilities.

CONCLUSION

These studies detail the development of tools for the production of highly active ST6Gal1, the preparation of anti-inflammatory disialylated Fc, and strategies to measure the structure and dynamics of glycoprotein glycans. The rates of sialylation and NMR spin relaxation prove to be informative of glycan position and dynamics.

ASSOCIATED CONTENT

Supporting Information

A supplementary figure showing the pulse sequences used for measuring the R_1, $R_{1\rho}$, and R_2 relaxation parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was financially supported by the grants R01GM033225 and P41RR005351 from the National Institutes of Health. A.W.B. was supported by a NIH Kirschstein National Research Service Award (F32AR058084).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Evan Brady for his preparation of the [$^{13}C_U$]-Gal and mono[$^{13}C_{1,2,3}$]-*N*-acetylneuraminic acid-labeled Fc.

ABBREVIATIONS

DC-SIGN, dendritic-cell specific ICAM3-grabbing nonintegrin; FcγR, Fcγ Receptor; Gal, galactose; GFP, green fluorescent protein; HEK, human embryonic kidney; HSQC, heteronuclear single quantum coherence; IgG, immunoglobulin G; Fc, fragment crystallizable; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; NMR, nuclear magnetic resonance; RA, rheumatoid arthritis; ST6Gal1, β-D-galactoside α2−6 sialyltransferase; TEV, tobacco etch virus.

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