Supporting Information

Experimental Procedures

Materials – All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise noted. CMP-^{13}C-[1,2,3]-*N*-acetylneuraminic acid was prepared according to Macnaughtan et al. (1), except unlabeled *N*-acetylmannosamine was condensed with ^{13}C-pyruvate (Cambridge Isotopes, Cambridge, MA) using *N*-acetylneuraminic acid aldolase.

Remodeling the Fc N-glycans – Pure immunoglobulin G (IgG) Fc fragment (Athens Research and Technology, Athens GA) was dialyzed overnight against water and lyophilized. Galactosylation of the N-glycan was achieved by resuspending the resulting material in 50 mM MOPS, 20 mM $MnCl_2$, 10 mM UDP-galactose, pH 7.2, to a concentration of 30 mg/ml Fc. This was followed by addition of 100 mU/mL bovine galactosyl transferase and incubation at 37°C for 24h. To ensure complete galactosylation, an additional aliquot of UDP-galactose and galactosyl transferase were added to the reaction and incubated at 37°C for an additional 24h.

The galactosylated Fc fragment was dialyzed overnight against 50 mM cacodylate, 10 mM $MnCl_2$, pH 6.0 and placed in a reaction with a final concentration of 50 mM cacodylate, 10 mM $MnCl_2$, 1 mM CMP-^{13}C-[1,2,3]-*N*-acetylneuraminic acid, 15 mg/ml Fc and 100 mU/mL α2-6 sialyltransferase (Calbiochem, San Diego, CA) at pH 6.0 and incubated at 37°C for 96h. An additional aliquot of CMP-^{13}C-[1,2,3]-*N*-acetylneuraminic acid and α2-6 sialyltransferase was added at 24, 48 and 72h during the incubation due to enzyme instability and the labile nature of the sugar nucleotide. Aliquots were analyzed at each step to assess the completeness of the reactions.

Glycan analysis by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI/TOF-MS) - N-glycans were released from the Fc fragment, purified as previously described (1) and

permethylated with NaOH and methyl iodide using the procedure of Anamula et al. (2). Permethylated glycans were crystallized and analyzed using MALDI/TOF-MS as previously described (1).

Exoglycosidase analysis of branch specificity – Fc fragment (1.65mg) was galactosylated and sialylated as described above, except galactosylation was only taken to ~90% completion. Fc was then purified using a Protein A – Sepharose column (GE Healthcare). N-glycans were released and purified as described above. Lyophilized, purified glycan was resuspended to a ~2 mg/mL in 50 mM sodium citrate, 100 mM NaCl, pH 6.0 to which 500 mU/mL β1-4 galactosidase (New England Biolabs, Ipswich, MA) was added. The reaction was incubated 16h at 37°C, then lyophilized. Degalactosylated glycan was resuspended in 100 μg/ml bovine serum albumin to which 100 mU/mL of β-*N*-acetylglucosaminidase (New England Biolabs) was added. The reaction was incubated for 16h at 37°C, then lyophilized. The resulting material was resuspended in 100 mM sodium acetate, 5 mM $CaCl_2$, pH 5.5, and 2.5 U/ mL of α1-2,3 mannosidase was added, then incubated for 16h at 37°C. Aliquots were analyzed at each step to assess the completeness of the reactions.

NMR analysis of branch specificity – Sialylated Fc fragment (1.5 mg) was purified using a Protein A - Sepharose Column (GE Healthcare). N-glycans were released and purified as described above, purified further using an OnGuard II H cation exchange column (Dionex, Sunnyvale, CA), lyophilized, resuspended in 99% D_2O, lypophilized, and finally resuspended in 25 mM $NaPO_4$, pH 7.0, prepared with 99.96% D_2O (Cambridge Isotopes). Roughly 30 μg of glycan was recovered. One-dimensional proton and two-dimensional COSY spectra were recorded at 5 and 25°C, a NOESY spectrum was recorded at 5°C and a TOCSY spectrum (Varian BioPak) was recorded at 25°C on a Varian 900 MHz spectrometer equipped with a cryogenically-cooled probe. Acquisitions required approximately 1 hr, 1d, 2d and 1d, respectively. Roughly 30 scans of each experiment were collected per minute. For the 2D experiments 1000 complex, indirect points over 7250 Hz were collected. Mixing times for the TOCSY and NOESY experiments were 80 ms and 300 ms, respectively.

Chemical shifts were referenced to an internal standard of 1 μM DSS (0.007 ppm). Spectra were processed using NMRPipe (3) and analyzed with NMRViewJ (4).

The H6′ proton of the galactose on the α1-3Man branch of the glycan was identified using an isotope filtered experiment in which ^{13}C-coupled spins were selected (5). The value of the three bond galactose H6′-^{13}C2 *N*-acetylneuraminic acid coupling was measured to be 2.5 Hz. The H6 proton, which likely overlaps with contaminating solute peaks, was not identified.

Sialylation of the released glycan – IgG Fc fragment (1.5 mg) was galactosylated and purified as described above. The N-glycans were released and purified as described in the *Glycan analysis by MALDI/TOF-MS* section. The released glycan was sialylated in 50 mM cacodylate, 10 mM $MnCl_2$, 1 mM CMP-*N*-acetylneuraminic acid, 1 mg/ml released glycan and 100 mU/mL α2-6 sialyltransferase (Calbiochem, San Diego, CA) at pH 6.0 and incubated at 37°C for 96h. An additional aliquot of CMP-*N*-acetylneuraminic acid and α2-6 sialyltransferase was added at 24, 48 and 72h. Aliquots were removed at 24h intervals and analyzed by MALDI/TOF-MS as described above. The ratios of MS peak volumes were recorded and were compared as a relative measure of glycoform abundance.

The released glycan used to determine ST6Gal-I branch specificity (Figure S1) was sialylated for only 16h, as a result of this relatively short time CMP-*N*-acetylneuraminic acid and α2-6 sialyltransferase was not replenished during the incubation.

Identification of GalNAc-modified Fc glycan- The preliminary identification of an *N*-acetylgalactosamine-modified glycan in the Fc glycan mixture was based upon the molecular weight observed in MALDI mass spectra and due to the apparent insensitivity of this glycoform to β-*N*-acetylglucosaminidase. This modification was observed in Figures 1, panels **C-F,** and S2, panels **A-C.**

Supporting Information References

1. Macnaughtan, M.A., Tian, F., Liu, S., Meng, L., Park, S., Azadi, P., Moremen, K.W., and Prestegard, J.H., (2008) C-13-sialic acid labeling of glycans on glycoproteins using ST6Gal-I. *J Am Chem Soc*, **130,** 11864-11865.
2. Anumula, K.R. and Taylor, P.B., A (1992) Comprehensive Procedure for Preparation of Partially Methylated Alditol Acetates from Glycoprotein Carbohydrates. *Anal Biochem*, **203**, p. 101-108.
3. Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A., (1995) NmrPipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *J Biomol NMR*, **6,** 277-293.
4. Johnson, B.A. and Blevins, R.A., NMRView - a Computer-Program for the Visualization and Analysis of NMR Data. *J Biomol NMR*, 1994. **4**, 603-614.
5. Cavanagh, J., (2007) *Protein NMR Spectroscopy : principles and practice*. 2nd ed., Amsterdam ; Boston: Academic Press.

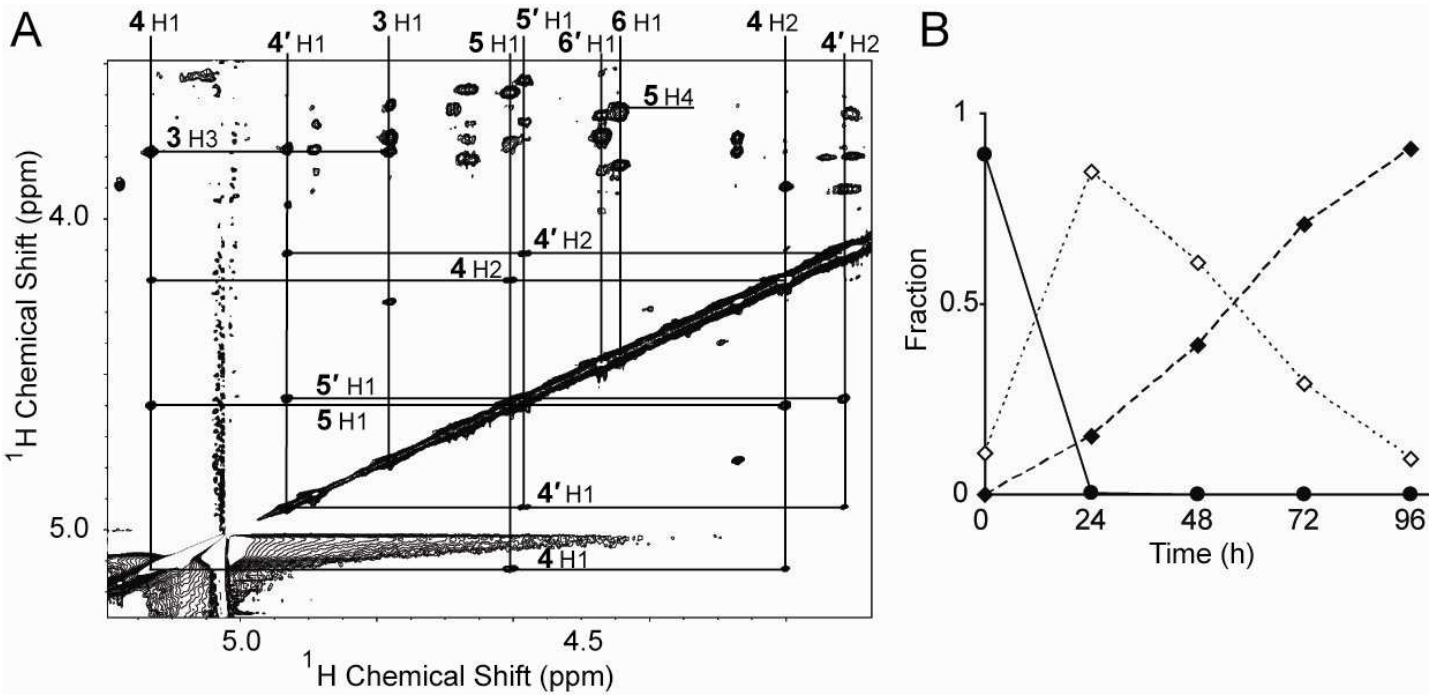

Figure S1 NMR based determination of glycan structures. (Panel **A**) A portion of a ^{1}H-^{1}H NOESY spectrum collected using a released monosialylated glycan shows anomeric connections and branch-specific residue assignments. (Panel **B**) A time course of released glycan sialylation using ST6Gal-I. The *closed* circles indicate a glycan with terminal galactose residues, *open* diamonds indicate a monosialylated glycan, and *closed* diamonds a disialylated glycan.

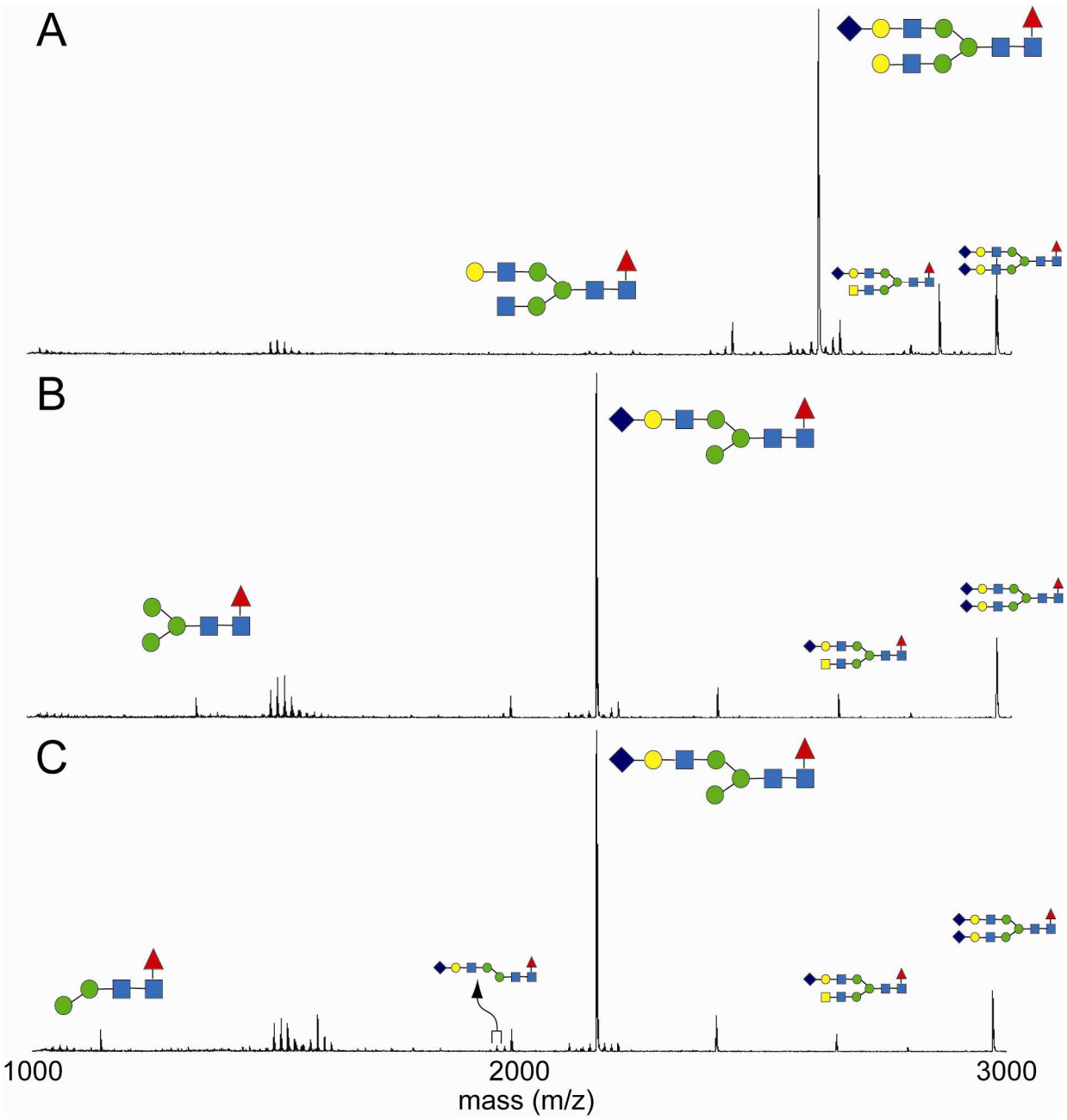

Figure S2 Sialylation of a released glycan results in *N*-acetylneuraminic acid primarily on the α1-3Man branch of the glycan. (Panel **A**) Following a 16 h sialylation reaction, the predominant glycoform contains one *N*-acetylneuraminic acid. (Panel **B**) Treatment with β-galactosidase and *N*-acetylglucosaminidase was sufficient to remove those residues not protected by a terminal sialic acid. (Panel **C**) The sialylated, β-galactosidase- and *N*-acetylglucosaminidase-treated glycan was not affected by an α1-2,3 mannosidase, suggesting the terminal mannose residue is α1-6 linked. The glycoform with two terminal mannose residues (mw 1346 in Panel B) was digested by the mannosidase in a manner consistent with the loss of hexose. Neither the disialyl glycoform or a glycoform consistent with that containing a terminal *N*-acetylgalactosamine residue were affected by the various treatments. The residue symbols are the same as those in Figure **1**.

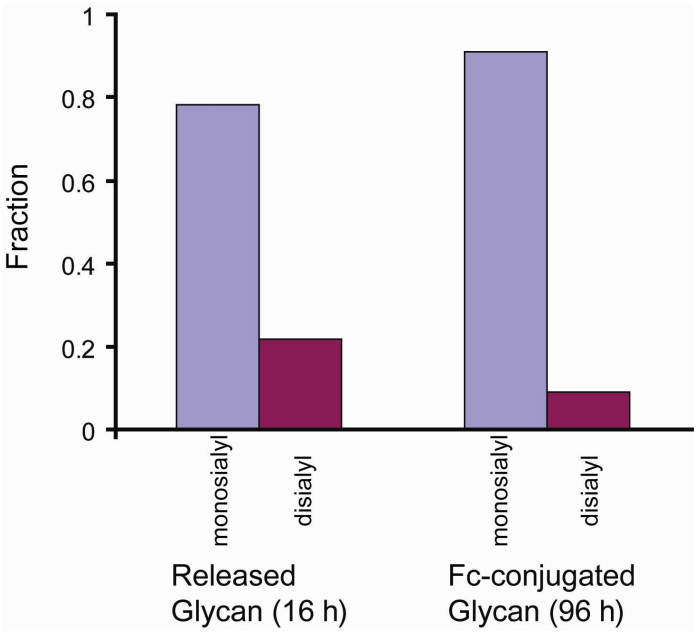

Figure S3. Sialylation of the released glycan compared to the Fc-conjugated glycan. Percentages of each isoform were calculated from MALDI-MS intensities

Table S1

Chemical shift values of the monosialylated glycan

0Fucose (alpha 1GlcNAc)		
6-deoxy-α-L-galactopyranose		
	H1	4.886
	H2	3.797
	H3	3.890
	H4	3.800
	H5	4.093
	H6	1.207
0Fucose (beta 1GlcNAc)		
6-deoxy-α-L-galactopyranose		
	H1	4.892
	H2	3.808
	H3	3.911
	H4	3.805
	H5	4.130
	H6	1.212
1GlcNAc (α)		
2-acetamide-2-deoxy-α-D-glucopyranose		
	H1	5.172
	H2	3.891
	H3	4.004
	H4	3.780
1GlcNAc (β)		
2-acetamide-2-deoxy-β-D-glucopyranose		
	H1	4.691
	H2	3.698
	H3	3.765
	H4	3.653
2GlcNAc		
2-acetamide-2-deoxy-β-D-glucopyranose		
	H1	4.662
	H2	3.785
	H3	3.594
	H4	3.747
3Mannose		
β-D-mannopyranose		
	H1	4.777
	H2	4.265
	H3	3.738
	H5	3.630
4Mannose		
α-D-mannopyranose		
	H1	5.127
	H2	4.195
	H3	3.894
	H4	3.510
4′Mannose		
α-D-mannopyranose		
	H1	4.921
	H2	4.107
	H3	3.892
5GlcNAc		
2-acetamide-2-deoxy-β-D-glucopyranose		
	H1	4.603
	H2	3.761
	H3	3.593
	H4	3.651
5′GlcNAc		
2-acetamide-2-deoxy-β-D-glucopyranose		
	H1	4.580
	H2	3.742
	H3	3.700
	H5	3.562
6Gal		
β-D-galactopyranose		
	H1	4.442
	H2	3.526
	H3	3.666
	H4	3.922
	H5	3.829
	H6′	3.980
6′Gal		
β-D-galactopyranose		
	H1	4.470
	H2	3.534
	H3	3.668
	H4	3.927
	H5	3.728
7NeuAc		
5-acetamide-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosonic acid		
	H3ax	1.713
	H3eq	2.660
	H4	3.694
	H5	3.657