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Production, Characterization, and Biological Evaluation of Well-Defined IgG1 Fc Glycoforms as a Model System for Biosimilarity Analysis

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ABSTRACT

Four different well-defined IgG1 Fc glycoforms are proposed as a model system to examine important biological and physicochemical features for protein drug biosimilar analyses. The IgG1 Fc glycoforms were produced by yeast expression combined with *in vitro* enzymatic synthesis as a series of sequentially truncated high-mannose IgG1 Fc glycoforms with an anticipated range of biological activity and structural stability. Initial characterization with mass spectrometry, SDS–PAGE, size exclusion HPLC, and capillary isoelectric focusing confirmed that the glycoproteins are overall highly similar with the only major difference being glycosylation state. Binding to the activating Fc receptor, FcγRIIIa was used to evaluate the potential biological activity of the IgG1 Fc glycoproteins. Two complementary methods using biolayer interferometry, 1 with protein G–immobilized IgG1 Fc and the other with streptavidin-immobilized FcγRIIIa, were developed to assess FcγRIIIa affinity in kinetic binding studies. The high-mannose IgG1 Fc and Man5-IgG1 Fc glycoforms were highly similar to one another with high affinity for FcγRIIIa, whereas GlcNAc-Fc had weak affinity, and the nonglycosylated N297Q-Fc had no measurable affinity for FcγRIIIa. These 4 IgG1 Fc glycoforms were also evaluated in terms of physical and chemical stability profiles and then used as a model system to mathematically assess overall biosimilarity, as described in a series of companion articles.

Introduction

Protein therapeutics are inherently structurally complex biological drugs whose active components are not a single well-defined molecule but a mixture of similar molecules that can differ by type and extent of post-translational modification,[1-5] chemical modifications,[6-11] and 3-dimensional conformations.[12,13] In addition, batch-to-batch variation of active components and production impurities can further complicate defining of the analytical characteristics of a protein drug.[14] Because of this, the assessment of protein therapeutics in comparability studies or in the regulatory approval of biosimilars is not just a simple exercise in confirming the chemical structure of a single active chemical entity in the presence of excipients. Instead, the primary and higher order structures, physicochemical properties, and biological activities of the protein therapeutics must be analyzed. Often, even after extensive study, the relationship between safety and efficacy of a protein therapeutic and these analytical tests is not entirely clear because of the complexity of the mixtures in protein therapeutics and biological systems involved. In an effort to better understand how *in vitro* analytical tests can be used to determine similarity in biosimilarity studies and analysis assessments, we have developed a series of well-defined IgG1 Fc glycoforms as a model system. The use of a series of well-defined glycoproteins in these studies should enable identification of important structural and biological features for comparability and biosimilarity analyses.

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A series of IgG1 Fc glycoforms were chosen as the protein model system for biosimilarity analysis because as a fragment of full-length IgG1 it is a simpler system to study but contains the C_H2 and C_H3 constant domains, which are present in all human IgG1-based mAb therapeutics. The Fc region is critical to antibody function in that it mediates effector functions, such as antibody-dependent cellular cytotoxicity (ADCC)[15-24] and complement-dependent cytotoxicity.[25-27] The Fc region of an IgG1 is also important in antibody clearance because binding of the Fc region to the neonatal Fc receptor (FcRn) increases *in vivo* half-life.[28-31] In addition, N-linked glycosylation at asparagine 297 (N297) in the Fc region is known to modulate the biological activity[19,32-39] and physical properties of IgG1 Fc,[1,32,40-48] and this can be used to establish similarities and differences between the members of this model system. A series of sequentially truncated glycoforms of IgG1 Fc (Fig. 1), which differ only in the size of the N-linked glycan at N297 or a single conservative amino acid mutation (N297Q), were chosen as members for the model system. Previous studies have indicated that these glycoforms would display a range of biological activities and physical properties[1,32,45] that would be advantageous for our biosimilarity studies.

It was first necessary to develop laboratory production methods to produce sufficient quantities (≈100 mg each) of the well-defined IgG1 Fc glycoforms to conduct the wide range of analytical tests necessary for biosimilarity analysis. Presented here are the methods established to produce the glycoforms shown in Figure 1 through yeast expression, purification, and *in vitro* enzymatic synthesis. Also presented in this work is the initial biochemical characterization of these glycoforms, the development of binding assays for IgG1 Fc binding to an Fc receptor using biolayer interferometry (BLI), and determination of the affinity of the different IgG1 Fc glycoforms for that Fc receptor as an initial evaluation of biological activity. Subsequent companion articles examine the physical and chemical stability profiles of these IgG1 Fc glycoforms, and the use of the resulting physicochemical data sets, to develop a mathematical algorithm for biosimilarity assessments.[49-51]

Materials and Methods

Materials

Yeast nitrogen base was obtained from Sunrise Biosciences, and Bacto™ Tryptone and Yeast Extract was purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Antifoam 204 was obtained from Sigma-Aldrich (St. Louis, MO). Certified American Chemical Society grade crystalline sucrose was purchased from Fisher Scientific (Pittsburg, PA). Boc-triglycine was supplied by Bachem Americas, Inc. (Torrance, CA). The enzymes PNGase F, Sortase, and *Bacteroides thetaiotaomicron* α-1,2-mannosidase (BT3990, *B.t.* α-1,2-mannosidase) were produced in-house.[52-55] Endoglycosidase H (Endo H) and restriction enzymes were obtained from New England Biolabs (Ipswich, MA). Protein G resin was produced by coupling protein G (recombinantly expressed in *Escherichia coli*)[56] with Sepharose® CL-4B (Sigma-Aldrich) using divinyl sulfone as a coupling reagent.[57] General chemicals were purchased from Sigma-Aldrich and Fisher Scientific unless otherwise noted.

Production and Initial Characterization of IgG1 Fc Glycoforms

Expression of High-Mannose IgG1 Fc

High-Mannose IgG1 Fc (HM-Fc) was expressed in a glycosylation-deficient strain of *Pichia pastoris* (an OCH1 and PNO1 deleted, IgG1 Fc expressing, SMD1168 strain of *P pastoris* produced by Xiao et al.[58] was used) using glycerol (growth phase) and methanol (induction phase) as carbon sources in an NBS BioFlo 415 fermenter (Eppendorf). The starter culture (2 mL) was allowed to grow at 25°C for about 72 h in YPD media (1% yeast extract, 2% peptone, 5% glucose, and zeocin 100 μg/mL). This starter culture was then inoculated into 250 mL of YPD media in a baffled shake flask and incubated for about 72 h. Before inoculation with the starter culture, the fermenter was filled with 7 L of fermentation basal salts medium (BSM).[59] The components of BSM per liter are 26.7 mL 85% phosphoric acid, 18.2 g potassium sulfate, 0.93 g calcium sulfate, 14.9 g magnesium sulphate heptahydrate, 4.13 g potassium hydroxide, and 40.0 g glycerol. Finally, water was added to the appropriate volume, and the fermentation media were sterilized. After sterilization and cooling, the temperature was adjusted to 30°C, and the pH of the BSM medium was adjusted to 6 with 28% ammonium hydroxide. Next, a trace minerals solution, PTM_1[59] salts solution, was prepared by mixing the following components (per liter): 6.0 g copper sulfate pentahydrate, 0.08 g sodium iodide, 3.0 g manganese sulfate monohydrate, 0.2 g sodium molybdate dihydrate, 0.02 g boric acid, 0.5 g cobalt(II) chloride, 20.0 g zinc chloride, 65.0 g iron(II) sulfate heptahydrate, 0.2 g biotin, and 5.0 mL sulfuric acid. This step was followed by adding 4.31 mL of filter-sterilized PTM_1 trace salts per liter of BSM. Next, the dense 250 mL starter culture media were added into the fermentor. The culture media were allowed to grow until the batch glycerol was consumed, and an additional 300 mL of glycerol were then added to increase the total cell biomass. Dissolved oxygen was kept at ≥30% throughout the fermentation processes. Once the added glycerol was consumed and the desired cell biomass was attained, a 100%

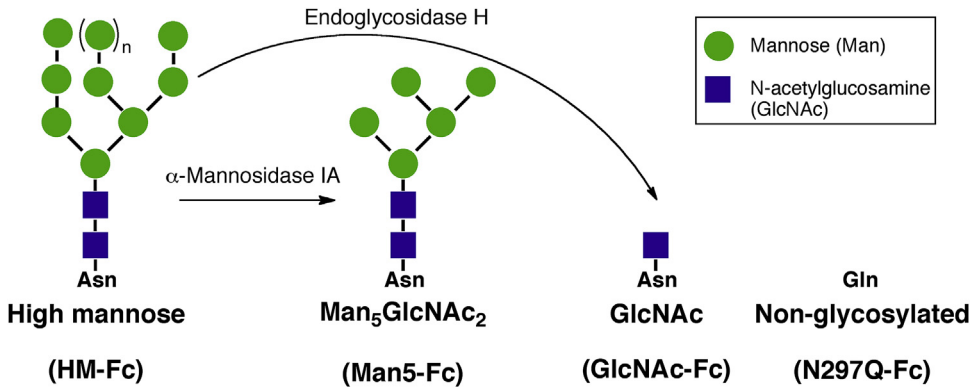

Figure 1. Production of homogenous IgG1 Fc glycoforms. HM-Fc was expressed in glycoengineered *P pastoris* and used as a precursor for generation of GlcNAc-Fc and Man5-Fc. N297Q-Fc was recombinantly expressed in a different yeast strain.

methanol feeding was initiated to induce the AOX1 promoter for IgG1 Fc expression. The temperature was reduced to 25°C for optimal IgG1 Fc expression before induction. Methanol induction was continued for approximately 72 h. The resulting yeast suspension was pelleted by centrifugation at 12,390 × *g* for 20 min, and the supernatant was collected. Using this procedure, the amount of HM-Fc obtained after protein G affinity purification was approximately 50 mg/L. Four 7-L fermentations were conducted to produce sufficient HM-Fc to develop the *in vitro* enzymatic synthesis used to make the Man5-Fc and GlcNAc-Fc glycoforms and to generate material for the subsequent experiments described in this and the accompanying articles.

Expression of Nonglycosylated Mutant N297Q-IgG1 Fc (N297Q-Fc)

Spinner flask expression was used for the production of N297Q-Fc in a glycosylation-deficient strain of SMD1168 *P pastoris* (clone produced in previous study[1]) as follows. A starter culture of 2 mL was inoculated in YPD media that contained 100 μg/mL Zeocin. This culture medium was incubated at 25°C for 72 h. The 2-mL culture was then used to inoculate 50 mL of YPD/Zeocin culture, which was incubated with shaking at 25°C for 72 h. The 50-mL culture was then used to inoculate 1 L of buffered glycerol–complex media (BMGY)[60] containing 0.00004% biotin and 0.004% histidine. After 48 h, when the initial glycerol carbon source was metabolized, N297Q-Fc expression was induced by methanol feeding to a final concentration of 1% (by addition of 50 mL of 20% methanol every 24 h) for about 72 h. The same protocol used for harvesting HM-Fc was applied for harvesting of the N297Q-Fc supernatant. Using this procedure, the amount of N297Q-Fc obtained after protein G affinity purification was about 20 mg/L. A total of seven 1-L spinner flasks were grown to produce 130 mg of N297Q-Fc used in the research described in this and the accompanying articles.

Purification of IgG1 Fc

Both HM-IgG1 Fc and N297Q-IgG1 Fc were purified using the same general procedure. The supernatant collected from yeast expression was filtered with 0.5-μm filter pads (Buon Vino Manufacturing) to remove particulates before protein G affinity chromatography. The protein G column (20-mL bed volume) was equilibrated with 20 mM potassium phosphate at pH 6.0 in 10 column bed volumes (CVs) and loaded with the filtered supernatant at pH 6.0. The column was subsequently washed with 20 mM potassium phosphate buffer, pH 6.0, containing 0.5 M NaCl (5 CV) and then 20 mM potassium phosphate buffer with a pH 6.0 (5 CV). IgG1 Fc was eluted by using 100 mM glycine at pH 2.7. Eluted protein was collected in 20-mL fractions into tubes containing 4 mL of 1 M Tris at pH 9.0 (200 μL of 1 M Tris at pH 9.0 per mL of elution volume) to neutralize the acidic elution buffer. Fractions of eluted protein detected by UV absorbance at 280 nm were immediately dialyzed in 20 mM sodium phosphate buffer at pH 7.0. Hydrophobic interaction chromatography (HIC) purification using phenyl sepharose™ high-performance resin (GE Healthcare) with a 125-mL CV (packed in-house) and an ÄKTAmicro chromatographic system (GE Healthcare) were used to further purify the IgG1 Fc forms. The phenyl sepharose column was pre-equilibrated with buffer A (20 mM sodium phosphate, pH 7.0, containing 1 M ammonium sulfate) for 5 CV. The protein G–purified IgG1 Fc was dialyzed in buffer A and then loaded onto the phenyl sepharose column with a loading volume of 50 mL (concentration, 1 mg/mL). The chromatographic separation was then conducted with 3 gradient segments from 0% to 50% buffer B (20 mM sodium phosphate, pH 7.0): gradient segment 1 (0%-25% B, 3.5 CV), gradient segment 2 (25%-30% B, 2.6 CV), and gradient segment 3 (30%-50% B, 6.7 CV). Collected fractions (10 mL) were analyzed by SDS–PAGE and mass spectrometry (MS) to check for purity and identity. Pure fractions were finally pooled and dialyzed in storage buffer (10% sucrose, 20 mM histidine at pH 6.0) and frozen at −80°C in aliquots. The final collected sample pool was analyzed by size exclusion HPLC (SEC), SDS–PAGE, MS, and capillary isoelectric focusing (cIEF) characterization to confirm overall purity and quality. Samples containing pure fractions were concentrated to 0.2 mg/mL using Vivaflow 50 (10,000 MWCO; Sartorius Stedim Biotech). Using this procedure, approximately 445 mg of the HM-Fc glycoform and 118 mg of the N297Q-Fc nonglycosylated mutant were produced for use in synthesis of other glycoforms and biosimilar analysis studies.

In Vitro *Enzymatic Synthesis of the Man5-IgG1 Fc Glycoform (Man5-Fc)*

HM-IgG1 Fc was converted to Man5-IgG1 Fc in an *in vitro* enzymatic reaction using *B.t.* α-1,2-mannosidase (BT3990).[54,55] It was found that the *B.t.* α-1,2-mannosidase used in this study had very low activity in the IgG1 Fc sample storage buffer, which contained 10% (w/v) sucrose and 20 mM histidine buffer at pH 6.0. Because of this, before the reaction, HM-Fc (125 mg) was dialyzed extensively in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 6.6 to remove the sucrose and histidine. Next, HM-Fc was dialyzed in a reaction buffer containing 5 mM $CaCl_2$, 150 mM NaCl, and 10 mM MES buffer at pH 6.6 for 12 h. After dialysis, the enzymatic reaction was started by adding 6.7 mg of the bacterial α-1,2-mannosidase (BT3990). The reaction was incubated at room temperature for 48 h. The progress of the reaction was monitored by MS, and the percentage of conversion to the Man5-Fc glycoform was estimated from the peak intensity (see Supplemental Table S1). Finally, the reaction mixture was purified using protein G affinity chromatography to remove unwanted impurities and excess enzyme using the same protocol as described in the purification of IgG1 Fc section as aforementioned. The amount of Man5-Fc produced was 75 mg (60% yield), and the percentage of Man5-Fc in the final product was estimated to be 78% by MS.

In Vitro *Enzymatic Synthesis of the GlcNAc-IgG1 Fc Glycoform (GlcNAc-Fc)*

HM-IgG1 Fc was converted to the GlcNAc-IgG1 Fc glycoform using Endo H. Endo H displayed full activity in the IgG1 Fc storage buffer (10% [w/v] sucrose, 20 mM histidine at pH 6.0); therefore, it was possible to digest HM-IgG1 Fc directly without a dialysis step. HM-IgG1 Fc (100 mg) at a concentration of 0.2 mg/mL was incubated with Endo H (for every 1 mg of HM-IgG1 Fc, 1000 U of Endo H enzyme were added, which corresponds to approximately 0.1 mg of Endo H per 100 mg HM-Fc) at room temperature for 24 h. The progress of the reaction was monitored by SDS–PAGE and MS. The sample was analyzed by MS, which showed a nearly quantitative reaction with the percentage of GlcNAc-Fc in the final product ≥99%.

LC–MS Analysis IgG1 Fc Glycoforms

Samples of IgG1 Fc glycoforms at a concentration of 0.2 mg/mL were first reduced with 10 mM dithiothreitol (DTT; Invitrogen, Carlsbad, CA), and then, 30 μL were injected into the mobile phase of the liquid chromatography (LC). Electrospray ionization spectra of the reduced samples were acquired on an Agilent 6520 Quadrupole Time-of-Flight system. The instrument was operated in positive ion mode, and a spectrum was acquired covering the mass range from 300 to 3000 m/z with an acquisition rate of 1 spectra/s. The samples were desalted on a reverse phase C4 column, 50-mm, 4.6-mm internal diameter (ID) (Vydac 214 MS; 300-A pore size, 5-μm particle size) using an Agilent 1200 series LC system. The solvents used were A (99.9% H_2O, 0.08% formic acid, and 0.02% trifluoroacetic acid) and B (99.9% acetonitrile, 0.08% formic acid, and 0.02% trifluoroacetic acid). A gradient was developed from 5% B to

90% B in 7 min with a flow rate of 0.5 mL/min. Data were collected using Agilent MassHunter Acquisition software (version B.02.00). Protein molecular weight (MW) was calculated using the maximum entropy deconvolution function, and associated peak intensities of the IgG1 Fc glycoforms were obtained using Agilent MassHunter Qualitative Analysis software (version B.03.01).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

For the reduced samples, each of the IgG1 Fc glycoforms (20 μg) were mixed with 2X TrisHCl SDS loading dye containing 100 mM DTT and incubated at 80°C for 2 min. The reduced IgG1 Fc samples were then separated by SDS–PAGE using NuPAGE 4%-12% Bis-Tris gradient gel (Life Technologies, Grand Island, NY) gels and an MES running buffer (Life Technologies). A similar method was followed for nonreduced samples of IgG1 Fc proteins except that DTT was omitted during the incubation step. The running time for all the gels was 60 min at 150 V. Protein bands were visualized by staining with Coomassie blue R250 (Teknova, Hollister, CA) and destained with a mixture of 30% methanol, 10% acetic acid, and 60% ultrapure water. Gel images were recorded using an Alphaimager (Protein Simple, Santa Clara, CA) gel imaging system.

Size Exclusion HPLC

Experiments were performed using a Shimadzu HPLC system equipped with a temperature-controlled auto sampler and a photodiode array detector capable of recording UV absorbance spectra from 200 to 400 nm. A Tosoh TSK-Gel Bioassist G3SW$_{XL}$ column (7.8 mm ID × 30.0 cm L) and a corresponding guard column (TOSOH Biosciences, King of Prussia, PA) were used for IgG1 Fc glycoform characterization. First, the SEC column was equilibrated for at least 10 CV with a mobile phase containing 200 mM sodium phosphate, pH 6.8, and a flow rate of 0.7 mL/min at 30°C column temperature. Next, the column was calibrated using gel filtration MW standards (Bio-Rad, Hercules, CA) before and after the runs of IgG1 Fc glycoform to ensure column and HPLC system integrity. All Fc samples were centrifuged at 14,000 *g* for 5 min before injection to remove insoluble protein aggregates. Protein samples at a concentration of 1 mg/mL were injected in a volume of 25 μL, and a 30-min run time was used for elution. Peak quantification was carried out using LC solutions software (Shimadzu, Kyoto, Japan). The error bars for monomer content for all the 4 IgG1 Fc samples and soluble dimer aggregates (observed in N297Q IgG1 Fc) represent SD of triplicate measurements.[1,61]

Capillary Isoelectric Focusing

The determination of isoelectric points (pIs) of all the IgG1 Fc glycoforms using cIEF were performed with an iCE280 analyzer from Convergent Biosciences (now Protein Simple, Toronto, Canada) equipped with a microinjector. An FC Cartridge (Protein Simple) with 50-mm and 100-μm ID fluorocarbon-coated capillary and built-in electrolyte tanks was used for focusing. The cartridge was calibrated by a hemoglobin standard (Protein Simple, Toronto, Canada) before and after focusing of IgG1 Fc samples to ensure its integrity. For focusing, a sample mixture was prepared where each of the IgG1 Fc glycoforms was mixed with urea (Fischer Scientific), methyl cellulose (Protein Simple), sucrose (Pfanstiehl Inc., Waukegan, IL), *N*,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine (Sigma-Aldrich), and Pharmalyte 3-10 (GE Healthcare Biosciences, Pittsburgh, PA). The final protein concentration in the sample mixture was 0.2 mg/mL. All the IgG1 Fc glycoforms were resolved using a prefocusing time of 1 min at 1500 V, and a focusing time of 12, 12, 7.5, and 7 min was used for HM-Fc, N297Q-Fc, GlcNAc-Fc, and Man5-Fc, respectively, at 3000 V. Observed peaks were calibrated using 2 pI markers with values of 5.84 and 8.18. The separation was monitored at 280 nm by a charge-coupled device detector. Quantitation of the peaks was done using Chromperfect® software (Justice Laboratory Software, Denville, NJ). The error bars for pI values of all the 4 IgG1 Fc samples represent SD of triplicate measurements.

Fc γ Receptor IIIa Binding Assays

Production of a P pastoris *Strain for Expression of Fc γ Receptor IIIa With a C-Terminal Sortase/Histidine Tag (FcγRIIIa-ST-H$_6$)*

The soluble region of the V158 polymorph of human FcγRIIIa was PCR amplified from pPICzαA-FcγRIIIa[58] using primers (forward 5′-ggcgccgaattcaaaagaatgcggactgaagatctc and reverse 5′-gccgcg cgcgcggccgcttaatgatgatggtggtggtgtccacctccagtttctggcaatccaccaccttg agtgatggtgatgttcac) that added a sortase recognition site (ST) and hexahistidine tag (H$_6$) to the 3′ end of the amplified FcγRIIIa DNA. The FcγRIIIa-ST-H$_6$ PCR product was inserted into the methanol-inducible Pichia expression vector pPICzαA (Invitrogen) using the EcoRI and Not I restriction sites. The pPICzαA-FcγRIIIa-ST-H$_6$ construct was confirmed by DNA sequencing, linearized using Sac I, and transformed into *P pastoris* OCH1 deleted cells.[58] Ten colonies were screened for levels of secreted FcγRIIIa-ST-H$_6$ expression by growing the colonies in 2-mL culture tubes containing BMGY[60] media + 100 μg/mL Zeocin at 25°C and 250 rpm. Once they reached density, 0.5% (v/v) methanol was added once per day for 3 days. Relative levels of FcγRIIIa-ST-H$_6$ in the media were determined by a dot blot using a mouse Anti-H$_6$ primary antibody (Thermo Scientific, Rockford, IL) followed by a goat Anti-mouse IgG secondary antibody conjugated with alkaline phosphatase (Thermo Scientific). The colony that expressed the highest level of FcγRIIIa-ST-H$_6$ was selected for 1-L spinner flask expression.

Expression and Purification of FcγRIIIa-H$_6$ and FcγRIIIa-ST-H$_6$

FcγRIIIa-H$_6$ and FcγRIIIa-ST-H$_6$ were expressed in glycosylation-deficient *P pastoris*, and Ni^{2+}-nitrilotriacetic acid (NTA) was used as described previously by Xiao et al.[58] After Ni^{2+}-NTA purification, approximately 30 mg/L of each receptor was obtained. The receptors were then further purified using HIC. A Hiprep™ phenyl FF (high sub) 16/10 column with a 20-mL bed volume was used with an ÄKTAmicro (GE Healthcare) to accomplish this. The column was pre-equilibrated with buffer A (1.5 M ammonium sulfate, 100 mM sodium phosphate for 10 CV) before loading. The Ni^{2+}-NTA purified receptors were first dialyzed in 100 mM sodium phosphate at pH 7.0 buffer for 12 h and then adjusted with ammonium sulfate to a final concentration of 1.5 M. The receptors in 10-mg portions were loaded onto the column with a loading volume of 5 mL. Because of the capacity of the column, 3 columns were necessary to purify all 30 mg of receptor obtained from Ni^{2+}-NTA chromatography. The chromatographic separation involves 3 segments from 0% to 100% B (100 mM sodium phosphate at pH 7.0): gradient segment 1 (0%-25% B, 15 CV), gradient segment 2 (25%-33% B, 15 CV), and gradient segment 3 (33%-100% B, 12 CV). Collected fractions (5 mL) were characterized using SDS–PAGE to check for purity. Samples containing pure receptor were concentrated to 1 mg/mL using Vivaflow 50 (10,000 MWCO, Sartorius Stedim Biotech), and the amount obtained after purification for each receptor was approximately 18 mg/L.

Biotinylation of FcγRIIIa-ST-H$_6$

Purified FcγRIIIa-ST-H$_6$ was extensively dialyzed in 50 mM Tris-hydrochloride at pH 7.5. Next, this receptor was dialyzed in a reaction buffer containing 50 mM Tris-hydrochloride at pH 7.5 and 150 mM sodium chloride. The sortase-mediated ligation reaction was carried out using a mixture containing 10 μM FcγRIIIa-ST-H$_6$, 6 mM $CaCl_2$, 1 mM GGG-linker-Biotin (compound 3, Fig. 2), and 5 μM

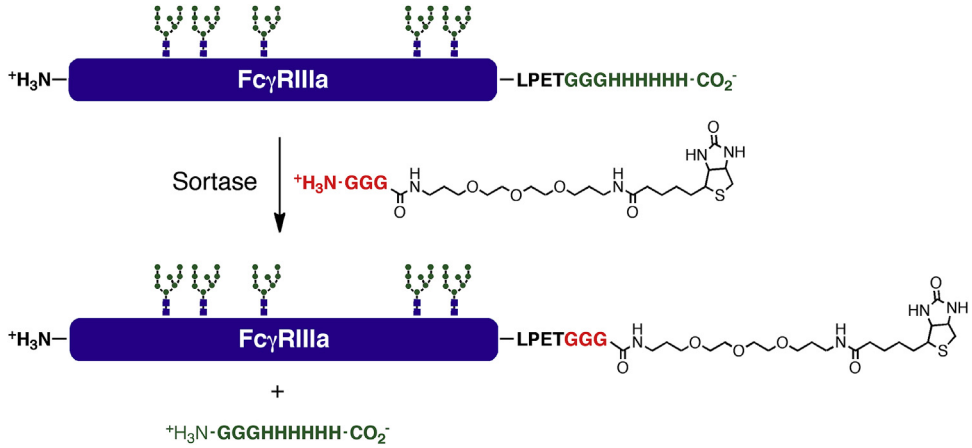

Figure 2. Production of biotinylated FcγRIIIa using sortase-mediated ligation between FcγRIIIa-ST-H_6 and GGG-linker-Biotin (compound 3 in the supporting information).

sortase at room temperature. The reaction was terminated after 24 h by adding excess ethylenediaminetetraacetic acid to capture the Ca^{2+} required for activity of the sortase. Finally, the receptor was extensively dialyzed in phosphate-buffered saline (PBS) buffer to remove the unreacted GGG-linker-Biotin (3).

Analysis of the Interaction of Immobilized IgG1 Fc Glycoforms With FcγRIIIa Using BLI

The interactions of the different IgG1 Fc glycoforms with the FcγRIIIa-H_6 were studied with BLI using a BLITZ instrument (Fortebio, Menlo Park, CA) with protein G biosensor tips. Binding studies using this receptor were conducted as follows. The protein G biosensor tip was hydrated for 10 min with PBS buffer (150 mM NaCl and 50 mM sodium phosphate at pH 7.4) and then incubated for 30 min with PBS kinetics buffer (PBS buffer containing 1 mg/mL casein as a blocking agent). Next, an initial baseline (30 s) was established with PBS kinetics buffer, and then, the protein G biosensor tips were loaded with the IgG1 Fc glycoforms at a concentration of 0.88 μM (120 s) to a response level of 2 nm. A new baseline (30 s) was then established, and then, the association (180 s) and dissociation (360 s) of FcγRIIIa-H_6 were measured by dipping the biosensor into solutions of FcγRIIIa-H_6 and PBS, respectively. To determine the dissociation constant (K_D) for the IgG1 Fc glycoforms, a range of FcγRIIIa-H_6 concentrations from 50 to 800 nM were tested for HM-Fc and Man5-Fc. For GlcNAc-Fc, the concentration range of FcγRIIIa-H_6 tested was 200-1600 nM in 2-fold serial dilutions. For N297Q-Fc, no binding was observed at 20 μM FcγRIIIa-H_6, the highest concentration of receptor tested. After each assay cycle, the biosensor tip was regenerated using 2 cycles of 10 mM HCl[62] for 30 s, and each time was equilibrated using PBS kinetics buffer for 60 s. Data generated from the binding of the receptor to IgG1 Fc glycoforms were collected 6 times and globally fitted to a 1:1 binding model and analyzed using BLITZ Pro software.

Analysis of the Interaction of IgG1 Fc Glycoforms With Immobilized FcγRIIIa Using BLI

The interactions of the different IgG1 Fc glycoforms with FcγRIIIa were studied with BLI using a BLITZ instrument (Fortebio, Menlo Park, CA) with streptavidin (SA) biosensor tips. Before the binding experiment, IgG1 Fc samples were dialyzed in PBS buffer to remove the storage buffer (10% sucrose, 20 mM histidine, and pH 6.0) and to adjust the pH to 7.4. The concentration of the samples after dialysis was 2.3 μM. Next, a stock solution of each glycoform in PBS kinetics buffer (PBS buffer containing 1 mg/mL casein) was prepared by adding casein (the stock solution of casein used was 10 mg/mL in PBS buffer) and PBS buffer. For HM-Fc and Man5-Fc, stock samples with a concentration of 1.6 μM were prepared and then serially diluted to prepare samples of 800, 400, 200, 100, and 50 nM concentrations by adding PBS kinetics buffer. For subsequent serial dilutions, PBS kinetics buffer containing 1 mg/mL of casein was used. Similarly, for GlcNAc-Fc, a stock sample with a concentration of 2.0 μM was prepared and then used to prepare samples of 1600, 800, 400, and 200 nM, by adding PBS kinetics buffer. For N297Q-Fc, after dialysis in PBS buffer, samples were first concentrated to 25 μM using an Amicon® Ultra-15 Centrifugal Filter Device with a MW cutoff of 10 kDa (EMD Millipore, Billerica, MA). Next, a solution containing 20 μM of N297Q-Fc in PBS kinetics buffer was prepared by dilution with PBS buffer and casein (added from a stock solution of 10 mg/mL casein in PBS buffer). This final sample concentration of 20 μM N297Q-Fc was then used for binding experiments without further dilution.

After sample preparation, binding studies were conducted as follows. First, the streptavidin biosensor tip was hydrated for 10 min with PBS buffer and incubated with PBS kinetics buffer for 30 min. Next, the biotinylated FcγRIIIa-ST-H_6 (0.1 μM) was immobilized onto the streptavidin biosensors to a response level of 0.4 nm, and this step was followed by establishing an initial baseline (30 s) with PBS kinetics buffer. Then, the association (180 s) and dissociation (360 s) of the IgG1 Fc glycoforms were measured by dipping the biosensor into solutions of IgG1 Fc glycoforms and PBS, respectively. After each assay cycle, the biosensor tip was regenerated using 2 cycles of 1 mM NaOH[63] for 30 s, and each time equilibrated with PBS kinetics buffer for 60 s. To determine the K_D, the concentration range tested for HM-Fc and Man5-Fc in solution was 50-800 nM in 2-fold serial dilutions. The concentrations of GlcNAc-Fc tested in solution ranged from 200 to 1600 nM in 2-fold serial dilutions. For N297Q-Fc, no binding was observed with 20 μM N297Q-Fc in solution. Data generated from the binding of the receptor to IgG1 Fc glycoforms were collected 6 times and globally fitted to a 1:1 binding model and analyzed using BLITZ Pro software.

Results

Production and Initial Characterization of the 4 Well-Defined IgG1 Fc Glycoforms

Expression and Purification of HM-Fc and Nonglycosylated Mutant N297Q-Fc

Both HM-Fc and N297Q-Fc were recombinantly expressed in glycosylation-deficient strains derived from SMD1168 *P pastoris* (Invitrogen). The expression strains were produced as described previously and have OCH1 and PNO1 genes deleted to reduce the

formation of higher order mannan structures and the addition of mannose phosphorylation.[1,58] The resulting yeast strains produce glycoproteins containing human-like high-mannose N-linked glycans with some additional heterogeneous mannose residues added onto an initial $Man_8GlcNAc_2$ structure. Because of the large amount of HM-Fc required for production of the 3 glycosylated forms, HM-Fc, Man5-Fc, and GlcNAc-Fc, the HM-Fc was produced in a 10-L fermentor using BSM supplemented with PTM_1 trace salts solution. Protein expression was induced by methanol addition, and yeasts were harvested after approximately 3 days of induction. Compared with HM-Fc, relatively smaller amounts of N297Q-Fc were required (because it is not used to produce the other Fc glycoforms). Accordingly, N297Q-Fc was expressed in spinner flasks using BMGY media to generate cell mass and 3 days of methanol induction to produce protein before harvest similar to the HM-Fc form. Typical yields from this expression system for the 2 IgG Fc proteins are summarized in the next section.

The same general purification procedure was used for both HM-Fc and N297Q-Fc. Yeast cells were removed by centrifugation, and the resulting supernatant was filtered through 0.5-μm filters to remove remaining particulates before chromatography. Secreted IgG1 Fc was then isolated by protein G affinity chromatography. The average yield of HM-Fc from fermenter growth after protein G affinity chromatography was approximately 50 mg/L. The average yield of N297Q-Fc after spinner flask expression was approximately 20 mg/L. After protein G affinity chromatography, there are still some residual yeast proteins remaining in both HM-Fc and N297Q-Fc. In the case of HM-Fc, incomplete glycosylation of the N297 site in yeast also results in macroheterogeneity of the glycosylation site.[1,2,58,64] Because of this, the disulfide-bonded HM-Fc dimer consists of 3 forms, a completely nonglycosylated form, a form that has glycosylation on only 1 chain of the dimer (monoglycosylated), and a form that is glycosylated on both chains of the dimer (diglycosylated). HIC using phenyl sepharose resin was used to remove residual yeast impurities for both proteins and to separate the diglycosylated form of HM-Fc from its monoglycosylated and nonglycosylated forms. Because protein G-purified HM-Fc is distributed between 3 different forms, the yield of diglycosylated HM-Fc from HIC purification is lower than that of N297Q-Fc. Nonetheless, after HIC purification, 445 mg of HM-Fc and 118 mg of N297Q-Fc were produced for further synthesis and studies. Hereafter, HM-Fc will refer to the diglycosylated form of HM-IgG1 Fc obtained from yeast. Purified protein samples were pooled, dialyzed in storage buffer (10% sucrose, 20 mM histidine, pH 6.0), concentrated or diluted to a final concentration of 0.2 mg/mL, and frozen at −80°C in aliquots for future use.

In Vitro *Enzymatic Synthesis of Man5-IgG1 Fc (Man5-Fc) and GlcNAc-Fc*

HM-Fc was converted into the Man5-Fc glycoform using *B.t.* α-1,2-mannosidase (BT3990).[54,55] The outer mannose residues of high-mannose N-linked glycans produced in glycosylation-deficient yeast are α-1,2-linked to a core of 5 mannose residues that are α-1,3- and α-1,6-linked to one another and β-1,4-linked to the 2 N-acetylglucosamines attached to asparagine. Because of this digestion, the HM-Fc glycoform with a selective α-1,2-mannosidase will result in the formation of the Man5-Fc glycoform. Unfortunately, initial trial mannosidase reactions revealed that the *B.t.* α-1,2-mannosidase had very little activity in the sucrose-containing storage buffer. Hence, before the reaction, the starting material (HM-Fc) was extensively dialyzed to remove the storage buffer and exchange it into a buffer the α-1,2-mannosidase had higher activity in. Addition of mannosidase (6.7 mg) and incubation at room temperature for 2 days resulted in conversion of the HM-Fc into the Man5-Fc glycoform. Protein G affinity chromatography was used to remove the mannosidase enzyme from Man5-Fc, and the protein was dialyzed into storage buffer and adjusted to 0.2 mg/mL concentration. A total of 75 mg of Man5-Fc was produced from 125 mg of HM-Fc starting material. The low yield of Man5-Fc is likely because of the many extra dialysis steps and the protein G purification in this procedure.

The enzyme Endo H was used to generate further truncation of the high-mannose glycan on HM-Fc. Endo H cleaves at the β-1,4 linkage between the 2 GlcNAc residues attached to asparagine in high-mannose N-linked glycans. This leaves a single GlcNAc monosaccharide attached at the glycosylation site (Fig. 1). Endo H has high activity in the sample storage buffer used to store HM-Fc, and this greatly simplified the production of the GlcNAc-Fc glycoform. A small amount ($\approx$0.1 mg) of Endo H added to 100 mg HM-Fc in storage buffer resulted in a quantitative conversion into the GlcNAc-Fc glycoform after incubation at room temperature for 24 h. Because Endo H was active in storage buffer and only a minute amount was added, GlcNAc-Fc was used without any further purification. Approximately 100 mg of GlcNAc-Fc was produced from 100 mg of HM-Fc starting material.

Analytical Characterization of IgG1 Fc Glycoforms

Figure 3 shows intact MS data of the 4 glycoforms, HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc, with their respective expected and observed masses. Because the amino acid sequences of HM-Fc, Man5-Fc, and GlcNAc-Fc are identical, the differences in observed masses are mainly because of differences in the attached N-linked glycans. For the HM-Fc glycoform, glycosylation is heterogeneous with high-mannose forms containing between 8 and 12 mannose residues with the major glycoform being the $Man_8GlcNAc_2$ form. The predominant forms of the truncated Man5-Fc and GlcNAc-Fc glycoforms on the other hand are largely 1 glycosylation state, with estimated abundance of 78% and 99% for the Man5 and GlcNAc forms, respectively, based on peak heights (see Supplemental Table S1). The N297Q-Fc form displays a single major peak corresponding to the nonglycosylated glutamine mutant as would be expected.

The 4 glycoforms were analyzed by SDS–PAGE under reducing and nonreducing conditions, and the results are shown in Figure 4. Purity was estimated to be ~99% for each glycoform, and no proteolysis or significant impurities were detected.[65] The shift in migration between the reduced and nonreduced gels indicates that intermolecular disulfide bonds are present forming dimers as would be expected in the hinge region. Also, no monomeric IgG1 Fc was detected in the nonreduced gel for any glycoform indicating that all the IgG1 Fc are present in the dimerized state. Migration of the different glycoforms in both the reduced and nonreduced gels correlates with the size of the N-linked glycans attached, with slower migration occurring for forms with larger N-linked glycans.

SEC was used to characterize the size and distribution of high-molecular weight species (HMWS) and low-molecular weight species (LMWS), which potentially could be generated during production and purification of IgG1 Fc glycoforms. HMWS could potentially be covalent aggregates, and LMWS are related to fragments from the heavy chains.[66,67] Representative SEC chromatograms of the IgG1 Fc glycoforms are shown in Figure 5 (also see Supplemental Table S2). All the IgG1 Fc proteins eluted at ~15 min, which corresponded to a monomer based on the elution pattern of MW standards. As illustrated in Figure 5, the results indicate that all the IgG1 Fc glycoforms are monomeric (96%-99%) with low levels of aggregates present across the IgG1 Fc samples (1%-3%). Low levels to essentially no HMWS were visible in the SEC chromatograms (less than estimated limit of quantification ~0.2%)[68] of all the 4 glycoforms except for the N297Q-Fc, which showed some soluble dimers (~3%). In addition, the SEC data also indicate low levels to

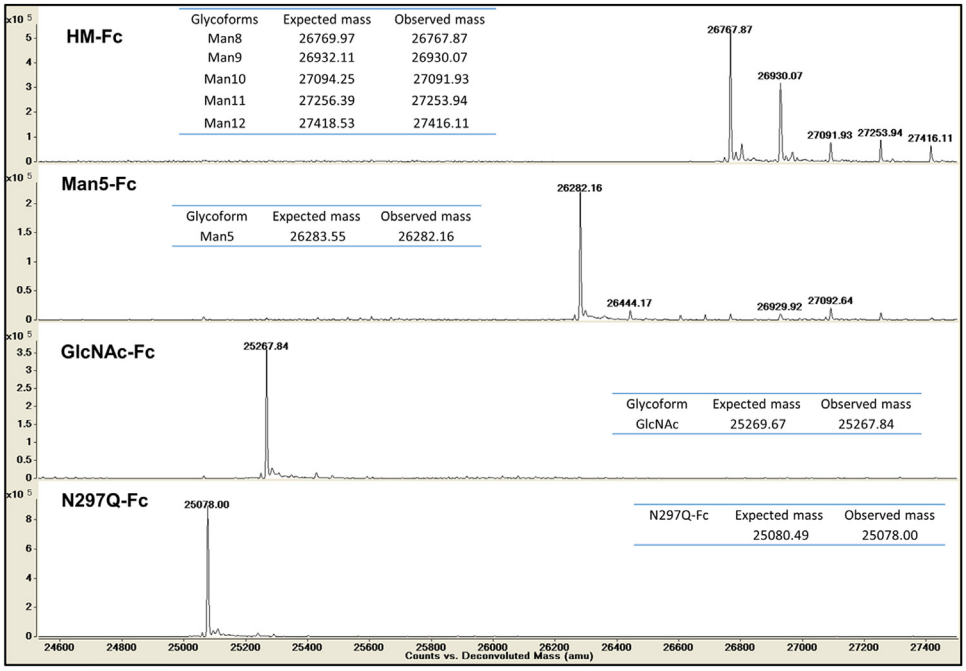

Figure 3. Intact protein MS analysis of IgG1 Fc glycoforms under reducing conditions.

essentially no LMWS, which is in agreement with SDS–PAGE characterization. All glycoforms were ≥98% monomeric except for the nonglycosylated form, which was greater than 96.4% monomeric. This difference is presumably because of the absence of glycosylation in N297Q-Fc. It has been reported that removal of glycosylation increases the aggregation propensity of IgGs.[1,45,47]

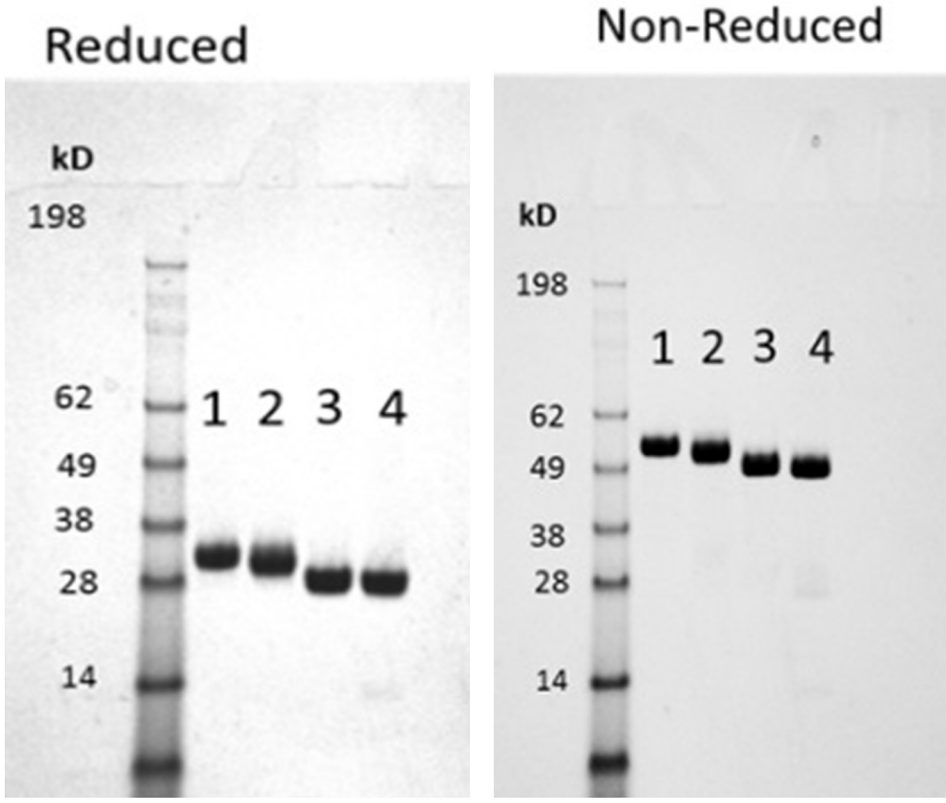

Figure 4. SDS–PAGE analysis of the 4 different IgG1 Fc glycoforms (1. HM; 2. Man5; 3. GlcNAc; and 4. N297Q) under reduced and nonreduced conditions. The purified IgG1 Fc glycoforms showed ~99% purity under both conditions. The HM-Fc glycoform that has the highest molecular weight runs slowest among the 4 types followed by the Man5-Fc, GlcNAc-Fc, and N297Q-Fc.

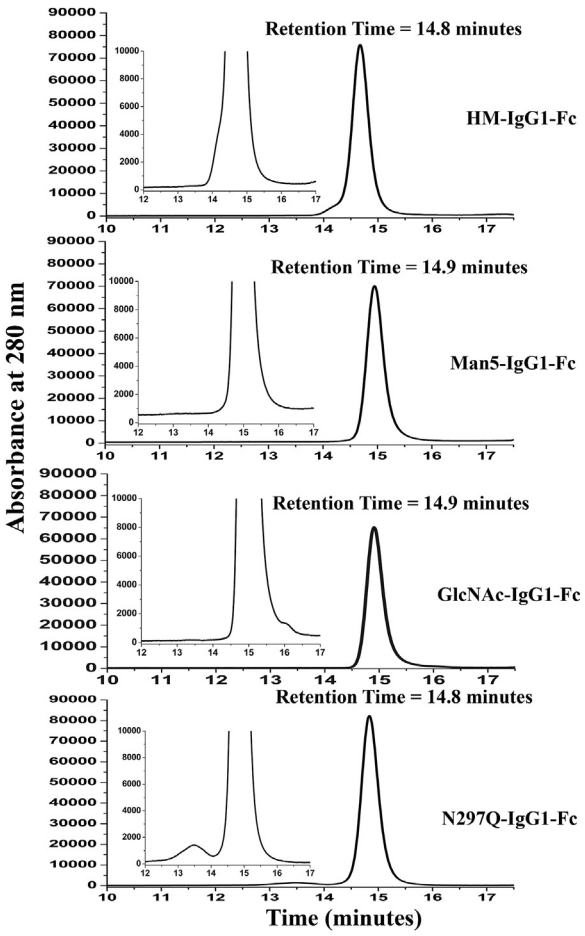

Figure 5. Representative size exclusion chromatograms of the IgG1 Fc glycoforms. Results showed the following total monomer content ($n = 3$; SD ~1.0%): 98.0% for HM-Fc, >99% purity for Man5-Fc and GlcNAc-Fc, and 96.7% for N297Q-Fc.

The charge distribution profiles of the 4 IgG1 Fc glycoforms were analyzed using cIEF. Representative electropherograms of HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc are shown in Figure 6 (also see Supplemental Table S3). All the electropherograms show 1 major peak, which after resolution gave pI values of 7.0, 6.9, 7.1, and 7.4 for HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc, respectively. The theoretical pI of IgG1 Fc from the amino acid sequence is 6.9, which is in close agreement with the experimental pI values obtained given assay variability. Nonetheless, the slightly basic shift of the aglycosyated form, compared with other 3 glycoforms, could be because of the lack of oligosaccharides at the N297 site. Although there are no visible basic variants observed, there are minor acidic peaks and shoulders near the main peaks with the pI values ranging from 5.6 to 6.8. The observed heterogeneity could potentially be from chemical modifications in IgG1 Fc proteins (e.g., deamidation) during production and/or purification.

In summary, these results demonstrate that each of the IgG1 Fc glycoforms is an overall well-defined glycoform species, with some minor charge heterogeneities and soluble aggregates present.

Evaluation of Affinity for IgG1 Fc Glycoforms With FcγRIIIa Using BLI

Expression and Purification of 2 Forms of FcγRIIIa for Use in Binding Assays

We have previously produced an expression strain for the production of the soluble domain of FcγRIIIa in yeast with a C-terminal histidine tag for affinity purification, reported by Xiao et al.,[58] and this strain was used to produce FcγRIIIa-H_6 used in the binding assays with IgG1 Fc immobilized on protein G biosensors. To reverse the immobilization format and have the receptor immobilized, a new expression strain was also constructed that produces the soluble domain of FcγRIIIa in yeast with a combined C-terminal histidine and sortase tag. This new receptor form, FcγRIIIa-ST-H_6, allows for affinity purification and the attachment of synthetic molecules, such as the synthetic biotin derivative used in this study, selectively to the C terminus using sortase-mediated ligation.[53,69-71] Both receptors were expressed in a glycosylation-deficient strain of *P pastoris* with glycerol and methanol as carbon sources. After centrifugation and filtration to remove yeast, Ni^{2+}-NTA affinity chromatography was used to isolate the FcγRIIIa forms. The receptors were further purified by phenyl sepharose chromatography, and after isolation and concentration, approximately 18 mg/L of purified protein were obtained for both forms of FcγRIIIa.

Selective C-Terminal Biotinylation of FcγRIIIa-ST-H_6 Using Sortase-Mediated Ligation

Sortase-mediated ligation[53,69-71] was used to attach biotin to the C terminus of FcγRIIIa. Bacterial sortases, such as the SrtA of *Staphylococcus aureus*, are enzymes that catalyze transpeptidase reactions to attach proteins containing sortase recognition sites to the peptidoglycan of bacterial cell walls. Sortases have been adapted for biotechnology applications by using the sortase reaction for immobilization and to attach synthetic molecules and labels selectively to the N- or C termini of recombinant proteins. The requirements for sortase ligations are N-terminal polyglycine-containing peptides, such as those found in peptidoglycan, and C-terminal recognition peptide motifs, such as LPETG, as in the SrtA-mediated ligation used in this study.[53,69-71] The C-terminal peptide is cleaved between threonine and glycine, and a new peptide bond is formed between the N-terminal polyglycine-containing molecule and the C-terminal threonine. Accordingly, to selectively biotinylate FcγRIIIa-ST-H_6, it was necessary to synthesize a form of biotin containing an N-terminal polyglycine. In addition, to ensure strong binding to streptavidin and prevent steric hindrance with streptavidin interfering with the interaction of FcγRIIIa with IgG1 Fc, a long hydrophilic diamine linker was used to separate biotin from the C terminus of FcγRIIIa. The synthesis of GGG-linker-Biotin (compound 3) is described in the supporting information for this article (Fig. S1), and the ligation of GGG-linker-Biotin to FcγRIIIa-ST-H_6 is shown in Figure 2. The sortase reaction proceeded efficiently to attach biotin to the C terminus of FcγRIIIa-ST-H_6, and the resulting biotinylated FcγRIIIa was immobilized in binding assays with streptavidin biosensors.

Analysis of Binding of Immobilized IgG1 Fc Glycoforms to FcγRIIIa Using BLI

The affinity of FcγRIIIa in solution for immobilized IgG1 Fc glycoforms was studied using BLI with protein G biosensors. The format for this binding study is shown in Figure 7a. Protein G biosensors were first loaded with IgG1 Fc glycoforms to a response level of 2 nm using 0.8-μM solutions of the various IgG1 Fc glycoforms. Then, a baseline was established and the association of FcγRIIIa was observed by dipping the biosensor into solutions of different concentrations of FcγRIIIa. After the Fc-FcγRIIIa complex had formed on the biosensor, dissociation of FcγRIIIa was observed by placing the biosensor in a solution of PBS kinetics buffer. The kinetic rate constants for association, k_a, and dissociation, k_d, were then obtained by fitting the resulting curves to a 1:1 binding model, which is consistent with previous biochemical and structural studies of this interaction.[72] The equilibrium dissociation constant

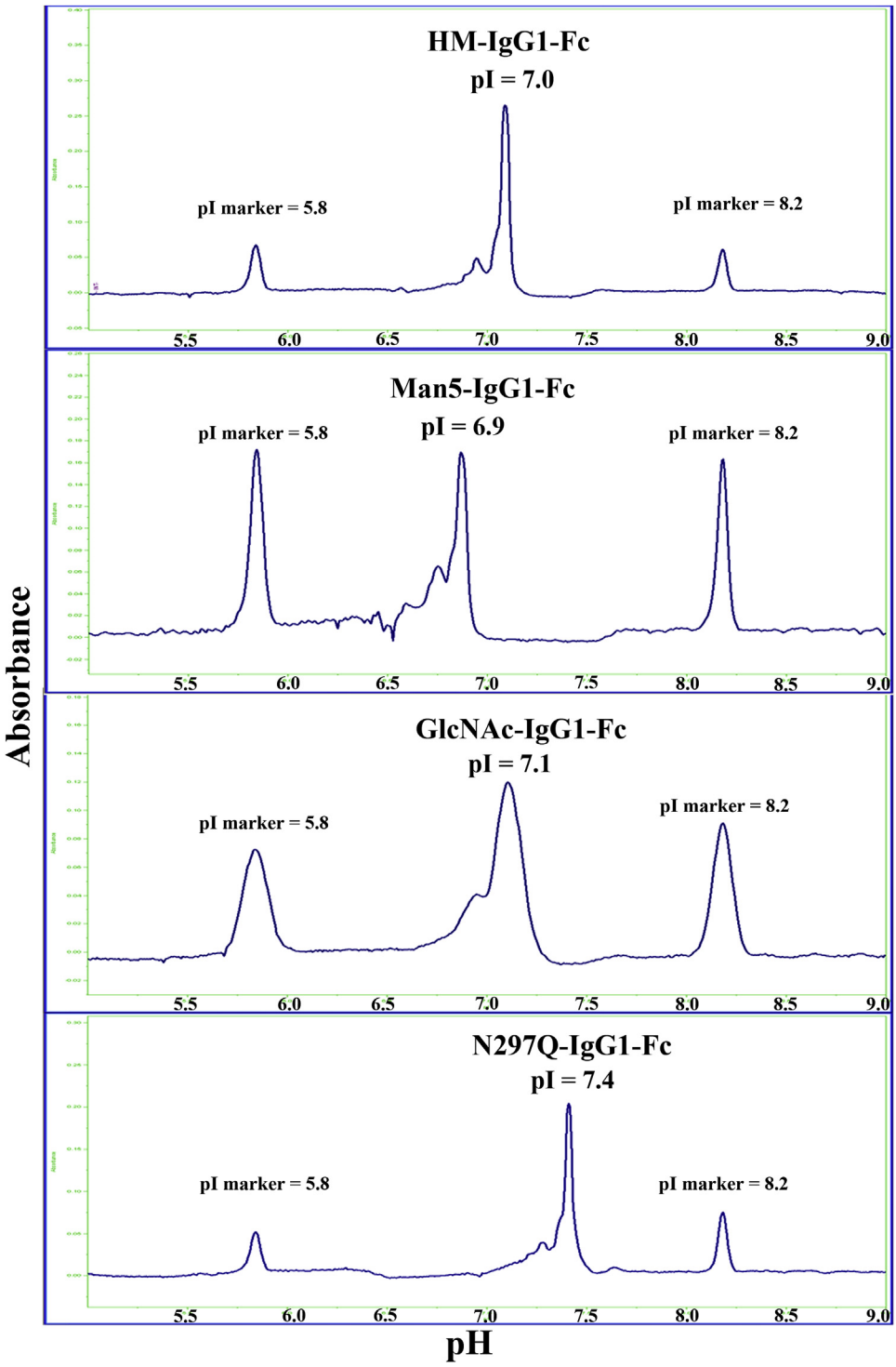

Figure 6. Representative charge heterogeneity profiles of IgG1 Fc glycoforms as measured by cIEF. The pI of the main peak ($n = 3$; SD ~0.1 pI units): pI of 7.0 for HM-Fc, pI of 6.9 for Man_5-Fc, pI of 7.1 for GlcNAc-Fc, and pI of 7.4 for N297Q-Fc.

was determined by dividing k_d by k_a for each glycoform. Representative sensorgrams for each glycoform (HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc) are displayed in Figure 8, and the results for these binding experiments are given in Table 1 (Supplemental Fig. S4 also displays representative sensorgrams with curve fits added). HM-Fc and Man5-Fc have similar high affinity for FcγRIIIa as would be expected for high mannose glycoforms, with K_Ds of 27.7 and 31.8 nM, respectively.[1,2,73] In contrast, the GlcNAc-Fc glycoform has significantly weaker binding to FcγRIIIa with a K_D of 1115 nM, and this agrees with recent studies of this glycoform.[74] Interestingly, the association rate constants (k_a) are very similar for HM-Fc, Man5-Fc, and GlcNAc-Fc, such that the driving factor behind the GlcNAc-Fc's weaker affinity for FcγRIIIa is its significantly faster dissociation rate (k_d). This similarity in k_a values and significant differences in k_d and K_D values is illustrated in Figure S3. No interaction was observed between FcγRIIIa and the

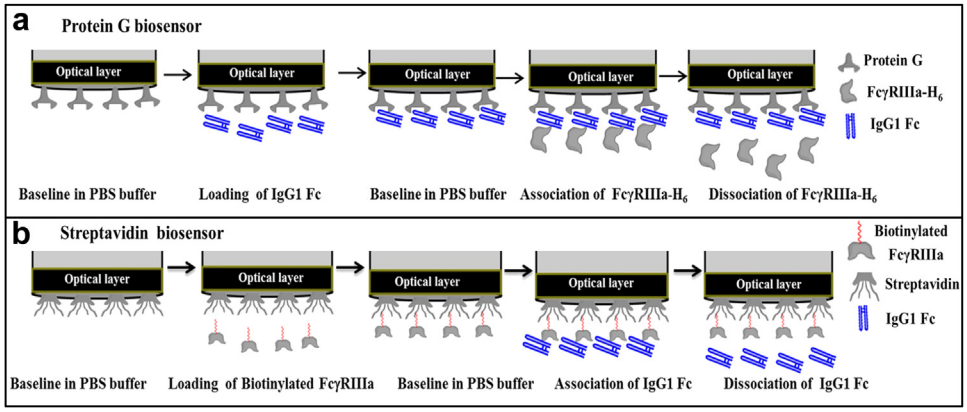

Figure 7. Steps followed in kinetic binding experiments using biolayer interferometry. (a) Top panel shows steps followed for measuring binding kinetics using protein G biosensors, where IgG1 Fc glycoforms were immobilized and FcγRIIIa-H_6 was present in solution. (b) Bottom panel shown steps involved in binding kinetics using streptavidin biosensor, where biotinylated FcγRIIIa was immobilized and IgG1 Fc glycoforms were present in solution.

nonglycosylated N297Q-Fc form at the highest concentration of receptor tested (20 μM).

Analysis of Binding of IgG1 Fc Glycoforms to Immobilized FcγRIIIa Using BLI

The affinity of the IgG1 Fc glycoforms in solution for immobilized FcγRIIIa was studied using BLI with streptavidin biosensors. The format for this binding study is shown in Figure 7b. Streptavidin biosensors were first loaded with C-terminally biotinylated FcγRIIIa to a response level of 0.4 using 0.1-μM biotinylated FcγRIIIa. Next, a baseline was established and the association of IgG1 Fc glycoforms was observed by dipping the biosensors into solutions of different concentrations of IgG1 Fc glycoforms. After the complex had formed on the biosensor, dissociation of the IgG1 Fc glycoforms was observed by dipping the biosensor into the PBS kinetics buffer. Kinetic rate constants and equilibrium dissociation constants were determined as described for the protein G method previously. Representative sensorgrams for each glycoform (HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc) are displayed in Figure 9, and the results for these binding experiments are given in Table 1 (Supplemental Fig. S5 also displays representative sensorgrams with curve fits added). Perhaps not surprisingly, the affinities determined for this receptor-immobilized binding format were very similar to the results observed for the IgG1 Fc–immobilized binding format. HM-Fc and Man5-Fc showed similar high affinity for FcγRIIIa with K_Ds of 26.4 and 32.8 nM, respectively.[1,2,73] Also, the GlcNAc-Fc glycoform displayed much weaker binding with a K_D of 995 nM, and this weak affinity was again because of a larger dissociation rate constant (k_d) for GlcNAc-Fc relative to the other glycoforms. Finally, the N297Q-Fc form displayed no affinity for immobilized FcγRIIIa even at 20 μM N297Q-Fc.

Discussion

Production and Initial Characterization of Well-Defined IgG1 Fc Glycoforms

As a first step to develop a model system for biosimilarity analysis studies, it was desirable to not only have a group of related well-defined proteins that differ from one another in specific attributes but also to be able to produce relatively large amounts of those proteins, on an academic laboratory scale, to provide sufficient material to conduct biosimilarity assessments through a combination of biochemical, physicochemical, and biological tests. To achieve this, glycosylation-deficient yeast (OCH1/PNO1 deleted *P pastoris* derived from strain SMD1168) that expresses human-like and high-mannose type N-linked glycosylation were used to produce the IgG1 Fc glycoproteins and the nonglycosylated mutant used in this and the accompanying studies. IgG1 Fc was secreted into culture media, and after centrifugation and filtration, the IgG1 Fc was captured and purified using protein G affinity chromatography. Residual yeast impurities and, in the case of HM-Fc, removal of mono- and nonglycosylated IgG1 Fc forms was accomplished using HIC chromatography. The nonglycosylated N297Q-Fc mutant (Fig. 1) was used directly as a negative control for the effects of glycosylation in these studies. The glycosylated HM-Fc glycoform (Fig. 1) is a member of this model system and also serves as the starting material for the production of the remaining 2 glycoforms of the model system. The HM-Fc glycoform was treated with *B.t.* α-1,2-mannosidase and Endo H to produce the Man5-Fc and

Table 1
Kinetic Parameters Obtained for Binding of FcγRIIIa With IgG1 Fc Glycoforms

Glycoforms	Immobilized Protein	Immobilization Technique	Average $k_a \times 10^{-5}$ (1/Ms)	Average $k_d \times 10^3$ (1/s)	Average K_D (nM)
HM-Fc	IgG1 Fc	Protein G	1.9 ± 0.3	5.4 ± 1.0	27.7 ± 6.4
	FcγRIIIa	Streptavidin	3.0 ± 0.6	7.9 ± 1.4	26.4 ± 6.9
Man5-Fc	IgG1 Fc	Protein G	1.6 ± 0.1	5.1 ± 0.9	31.8 ± 5.9
	FcγRIIIa	Streptavidin	2.2 ± 0.2	7.3 ± 0.3	32.8 ± 3.1
GlcNAc-Fc	IgG1 Fc	Protein G	1.2 ± 0.2	140.0 ± 9.1	1115.0 ± 136.8
	FcγRIIIa	Streptavidin	1.6 ± 0.3	161.3 ± 21.8	995.0 ± 219.3
N297Q-Fc	IgG1 Fc/FcγRIIIa	Protein G/streptavidin	[a]	[a]	[a]

Binding kinetics was measured at 25°C. The data show kinetic association rate k_a, kinetic dissociation rate k_d, and equilibrium dissociation constant K_D. These data are averaged values of 6 independent experiments.

[a] For N297Q, there was no detectable binding at the highest concentration tested (20 μM) for both methods.

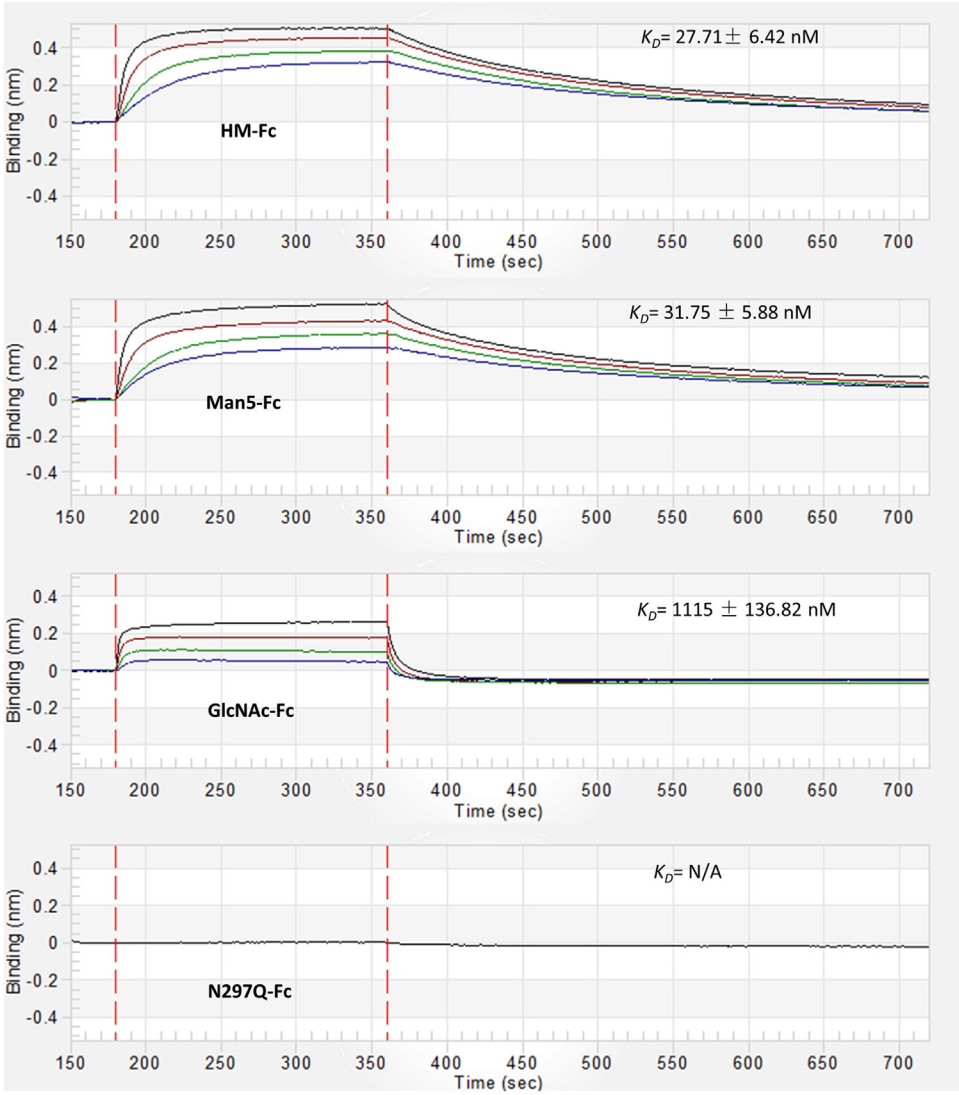

Figure 8. Representative BLI binding data for the interaction of FcγRIIIa with IgG1 Fc glycoforms immobilized on protein G biosensors. The binding curves correspond to a range of receptor concentrations in solution. For HM-Fc and Man5-Fc, data are shown for FcγRIIIa at concentrations of 800, 400, 200, and 100 nM, which corresponds with the curves from top to bottom. For GlcNAc-Fc, data are shown for FcγRIIIa at concentrations of 1600, 800, 400, and 200 nM, which corresponds with the binding curves from top to bottom. For N297Q-Fc, a single concentration of 20 μM was tested where no binding was observed.

GlcNAc-Fc glycoforms, respectively (Fig. 1). The HM-Fc, Man5-Fc, and GlcNAc-Fc glycoforms form a well-defined series where HM-Fc contains the largest N-linked glycan, and the remaining glycoforms are sequentially truncated to give the intermediate glycosylation state of Man5-Fc and the minimally glycosylated state of GlcNAc-Fc. This series of glycoproteins with decreasing N-linked glycan size derived from the same precursor should be useful in elucidating effects of glycosylation on the biological and physicochemical properties of IgG1 Fc.

In terms of ease of production, the HM-Fc and N297Q-Fc are both derived directly from fermentation and therefore more easily accessed than the other 2 glycoforms. Because N297Q-Fc has no glycosylation site occupancy heterogeneity, its HIC purification is simplified compared with the HM-Fc form, which requires separation of di-, mono-, and nonglycosylated forms. After HIC purification of both forms, 445 mg of HM-Fc and 118 mg of N297Q-Fc were produced for this study from approximately 28 and 7 L of growth media, respectively. Conversion of 100 mg of HM-Fc into GlcNAc-Fc using Endo H (approximately 0.1 mg) was straightforward and nearly quantitative, allowing relatively easy access to 100 mg of the GlcNAc-Fc glycoform. Production of the Man5-Fc glycoform was more problematic because it was discovered that the *B.t.* α-1,2-mannosidase had little activity in sucrose-containing storage buffer after the HM-Fc had been transferred into the storage buffer. This required additional dialysis steps to transfer the HM-Fc into a different buffer to conduct the *B.t.* α-1,2-mannosidase reaction. In addition, a larger amount of *B.t.* α-1,2-mannosidase (6.7 mg) relative to Endo H (0.1 mg) was required for the reaction to produce Man5-Fc, and this made it necessary to purify the Man5-Fc glycoform by an additional protein G affinity chromatography step after the mannosidase reaction. All these factors combined to result in a 60% yield for conversion of HM-Fc into Man5-Fc and only 75 mg of the Man5-Fc glycoform being produced. The amount of Man5-Fc produced was sufficient to conduct this and the accompanying studies, but the procedure to produce this glycoform could be optimized in the future to produce more of this glycoform in a higher yield. Taken together, these methods allow easy access to the 4 well-defined glycoforms from Figure 1 in quantities sufficient to enable a wide range of biosimilar analysis and stability studies.

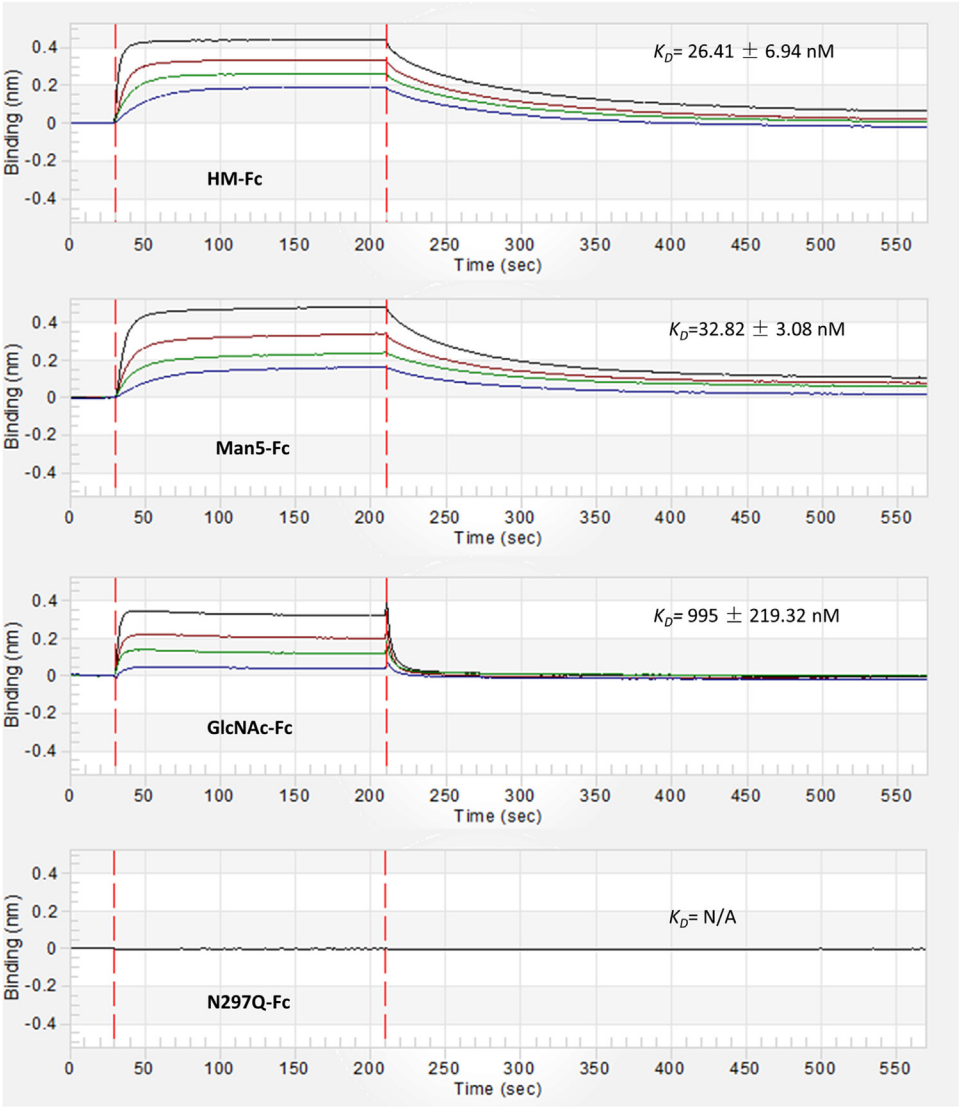

Figure 9. Representative BLI binding data for the interaction of IgG1 Fc glycoforms with FcγRIIIa immobilized on streptavidin biosensors. The binding curves correspond to a range of IgG1 Fc glycoform concentrations in solution. For HM-Fc and Man5-Fc, data are shown for IgG1 Fc glycoforms at concentrations of 800, 400, 200, and 100 nM, which corresponds with the curves from top to bottom. For GlcNAc-Fc, data are shown for GlcNAc-Fc at concentrations of 1600, 800, 400, and 200 nM, which corresponds with the binding curves from top to bottom. For N297Q-Fc, a single concentration of 20 μM was tested where no binding was observed.

Initial characterization of the glycosylation state of the model glycoforms was conducted using intact protein MS. MS was used to confirm the type of glycosylation on each glycoform and determine if there were any significant proteolytic products present (Fig. 3). None of the IgG1 Fc glycoforms showed any evidence of internal proteolysis, and all were found to be in forms containing their complete amino acid sequences, including C-terminal lysine residues. The intact protein mass spectra also allowed evaluation of the glycosylation state of the IgG1 Fc glycoforms. The N297Q-Fc and GlcNAc-Fc glycoforms were highly homogeneous with single peaks corresponding to no glycosylation with a glutamine mutation and a single GlcNAc attached to asparagines, respectively. The HM-Fc glycoform obtained directly from yeast was heterogenously glycosylated with high-mannose type glycans ranging in size from $Man_8GlcNAc_2$ to $Man_{12}GlcNAc_2$, with the form containing $Man_8GlcNAc_2$ being the major form at approximately 49% of its glycosylation sites based on peak intensities (supporting information, Supplemental Table S1). The mannosidase-treated Man5-Fc glycoform is much more homogeneous and estimated to contain $Man_5GlcNAc_2$ on 78% of its glycosylation sites based on peak intensities (supporting information, Supplemental Table S1). The remainder of the glycoform peaks observed in the Man5-Fc mass spectra corresponds to small amounts of incompletely cleaved high-mannose glycosylated forms, indicating that the mannosidase reaction could be further optimized in the future. The MS results demonstrate well-defined differences between the model IgG1 Fc glycoforms based on the size or the absence of N-linked glycosylation.

Additional biochemical characterization was conducted using SDS–PAGE, SEC, and cIEF. This was done to further define the chemical state and purity of the IgG1 Fc glycoforms and to establish a baseline before the chemical and physical stability studies described in the companion articles in this series. Reducing and nonreducing SDS–PAGE (Fig. 4) display an absence of proteolytic fragments and show high purity for each glycoform. The electrophoretic mobility of the IgG1 Fc glycoforms shown in Figure 4 was also consistent with slower migration for the larger branched N-linked glycoproteins with HM-Fc migrating slowest,

Man5-Fc migrating intermediately, and the GlcNAc-Fc and N297Q-Fc forms migrating fastest.[75] In addition, the nonreduced SDS–PAGE indicated complete intermolecular disulfide bond formation corresponding to 100% dimerized form for all glycoforms. As illustrated in Figure 5, the SEC results indicate that the IgG1 Fc glycoforms are mostly monomeric (96%-99%) with low levels of aggregates present across the IgG1 Fc samples (1%-3%). Low levels to essentially no HMWS were visible in the SEC chromatograms (less than estimated limit of quantification ~0.2%) of all the 4 glycoforms except for the N297Q-Fc, which showed some soluble dimers (~3%). In addition, the SEC data also indicate low levels to essentially no LMWS, which is in agreement with SDS–PAGE characterization. In summary, the Fc glycoforms were ≥98% monomeric by SEC except for the nonglycosylated form, which was 96.4% monomeric. This difference is presumably because of the absence of glycosylation in N297Q-Fc. It has been reported that removal of N-linked glycans in the Fc region increases the aggregation propensity of IgG Fcs and IgGs.[1,45]

Analysis of the pIs for the 4 glycoforms by cIEF identified a difference between the N297Q-Fc form and the other forms, with the N297Q-Fc form having a pI of 7.4 compared with 7.0, 6.9, and 7.1 for HM-Fc, Man5-Fc, and GlcNAc-Fc, respectively (Fig. 6). Based on MS analysis described in the companion article in this series,[50] the only Asn residue that displayed measurable deamidation in the 4 nonstressed Fc samples was N315 (0.5%-2.0%), but the levels were similar for the Man5 glycoform and the N297Q aglycosylated form (~0.5%; data not shown); yet, their pI values were different. Thus, Asn deamidation is not the likely cause of the observed differences. Moreover, the N-linked glycans present on the glycoforms are neutral glycans and are not charged. Interestingly, it has been noted by our laboratory and others that cation exchange chromatography can be used to separate glycosylated and nonglycosylated proteins from one another even when only neutral oligosaccharides are present on the glycosylation sites. The elution order observed from cation exchange chromatography in the cases of separation of glycosylated and nonglycosylated IL-1ra and diglycosylated IgG from mono- and nonglycosylated IgG is consistent with the nonglycosylated forms having higher pIs.[2,3,76] It may be that the measured pI differences arise from different interactions of the IgG1 Fc glycoforms within the polymeric cartridge containing ampholytes used in cIEF, rather than actual pI differences. The root cause of the observed pI differences appears to be complex and will be a topic for future studies.

Biological Evaluation of Well-Defined IgG1 Fc Glycoforms

Potential biological activities of the IgG1 Fc glycoforms in our model system were assessed using binding to an activating Fc receptor, FcγRIIIa, as a measure of the potential to activate antibody-dependent effector functions. In full-length antibodies, simultaneous binding of the antigen-binding regions of an antibody to a polyvalent antigen and the antibody Fc region to FcγRIIIa can activate ADCC.[77] FcγRIIIa is also important in the function of natural killer (NK) cells because it is the only Fc receptor expressed on NK cells.[78] Both ADCC and NK cells are believed to be important in the function of many therapeutic mAbs.[78,79] In addition, FcγRIIIa is sensitive to the type of glycosylation present on the Fc region of antibodies, having high affinity to antibodies that do not contain core-linked fucose, and like most Fc receptors, lower affinity for truncated N-linked glycans.[79] This makes FcγRIIIa useful in distinguishing the different IgG1 Fc glycoforms in our model system.

The interactions of the IgG1 Fc glycoforms with FcγRIIIa were assessed with kinetic binding studies using BLI. Observation of binding using surface techniques, such as BLI or surface plasmon resonance (SPR), generally does not require large amounts of material to conduct binding studies, and this is an advantage for biosimilarity analysis studies where many samples may be required to be analyzed. Kinetic binding studies using BLI or SPR allow determination of kinetic rate constants for association (k_a) and dissociation (k_d), and by taking the ratio of the kinetic rate constants, equilibrium dissociation constants (K_D) can be determined. One drawback of surface binding techniques is that 1 binding partner, receptor or ligand, needs to be immobilized to conduct the experiment (Fig. 7). Choice of which component, receptor or ligand, to immobilize and method of immobilization can both affect the outcome of the experiment. In addition, from the perspective of conducting experiments with samples of formulated drug products, the format of binding assays can have practical effects on how the assays are conducted and what kind of information is obtained. To gain a better understanding of how binding format affects the results of binding studies, we developed 2 formats for studying the interaction of IgG1 Fc glycoforms with FcγRIIIa (Fig. 7), 1 with the IgG1 Fc immobilized and 1 with the receptor immobilized.

For a BLI binding format where the IgG1 Fc glycoforms were immobilized (Fig. 7a), we decided to use protein G biosensors for immobilization of IgG1 Fc glycoforms. Protein A and/or protein G have previously been used in SPR binding studies of IgG interactions with Fc receptors, and immobilization using these proteins has many advantages.[80,81] Because protein G binds tightly to IgGs over a wide pH range (≈pH 5-8), protein G biosensor tips can be loaded under a variety of formulation conditions without having to adjust the buffer conditions of samples to promote immobilization. In addition, once IgG1 Fc (or full-length IgG) has been immobilized onto a protein G biosensor, binding experiments can be conducted in buffers optimized for receptor binding by simply dipping the IgG1 Fc–loaded protein G biosensor into receptor solution with optimized buffer conditions. Also, protein G biosensors can be regenerated by acidic treatment much the way protein A and protein G affinity resins are eluted during affinity chromatography, allowing for relatively easy reuse of protein G biosensors. A disadvantage of using protein G biosensors to immobilize IgG1 Fc in binding studies may arise during stability studies when there is the potential of the formation of damaged forms of IgG1 Fc, which would no longer be capable of binding to protein G, but which could still retain the ability to bind to Fc receptors. Such proteins would not be measured in this assay format. Also, this assay format is relatively insensitive to the actual concentration of IgG1 Fc glycoforms in samples because to obtain reproducible binding curves the biosensors are loaded to the same approximate level, and because of this, changes in concentrations in the samples may be difficult to detect. Nevertheless, this assay format provides valuable information about the kinetics and thermodynamics of the interaction of IgG1 Fc glycoforms with FcγRIIIa.

Figure 8 shows representative binding curves, and Table 1 summarizes the resulting rate constants and dissociation constants obtained for the interaction of immobilized IgG1 Fc glycoforms with FcγRIIIa in solution using this binding format. As can be seen in Table 1, both HM-Fc and Man5-Fc have high affinity for FcγRIIIa (K_Ds of 28 and 32 nM, respectively), GlcNAc-Fc showed much lower affinity (K_D of 1115 nM), and N297Q-Fc had no observable affinity for FcγRIIIa (all measurements were conducted at 25°C). Examination of the rate constants for the different interactions reveals that the main factor in the GlcNAc-Fc glycoform's lower affinity is the significantly faster dissociation of the complex once it forms relative to HM-Fc and Man5-Fc, and this can be seen in the magnitudes of the dissociation rate constants, k_d (Table 1), and the ratios of kinetic and thermodynamic values when compared with HM-Fc values (supporting information, Supplemental Fig. S3).

For a BLI binding format where FcγRIIIa is immobilized and IgG1 Fc glycoforms were in solution (Fig. 7b), we used selectively

biotinylated FcγRIIIa and streptavidin biosensors. Streptavidin biosensors were chosen for immobilization of FcγRIIIa because the high affinity of nonfucosylated IgG1 Fcs, such as HM-Fc and Man5-Fc ($K_D \approx 30$ nM), required either covalent or significantly higher affinity immobilization to prevent artifacts related to dissociation of receptors during binding measurements. The high affinity of streptavidin for biotin ($K_D \approx 10^{-14}$ M)[82] was appropriate for this application; therefore, we developed a novel method to selectively biotinylate the C terminus of FcγRIIIa using sortase-mediated ligation. This required constructing an expression strain of FcγRIIIa, which contained a C-terminal sortase tag, expression and purification of the sortase-tagged FcγRIIIa, synthesis of a triglycine-containing biotin derivative (compound 3, supporting information), and sortase-mediated ligation to produce the C-terminally biotinylated FcγRIIIa, and these methods are described in the supporting information. An advantage of using an immobilized FcγRIIIa binding format such as that shown in Figure 7b is that binding measurements can be made directly in solutions of IgG1 Fc glycoforms. This type of format is also potentially more sensitive to changes in IgG1 Fc concentration because measurements are done with the samples directly in solution rather than in an immobilized format.

A disadvantage of this binding format is that buffer conditions present in samples must be compatible with receptor binding measurements. The affinity of many receptors is pH dependent, so formulation pH could affect binding measurements. In addition, high concentrations of sugars, such as the 10% sucrose in our sample storage buffer, can have significant effects on the kinetics and thermodynamics of binding measurements. Because of these factors, in our experiments, it was necessary to transfer the IgG1 Fc glycoforms from storage buffer (10% sucrose and 20 mM histidine at pH 6.0) to kinetics buffer (PBS buffer containing 1 mg/mL casein as a blocking agent) by dialysis, before binding measurements. This added a step to the binding measurements and has the potential to skew results if there is loss of sample or changes in concentration during the transfer of samples to the new buffer. The results of the receptor-immobilized binding format are shown in Figure 9 and Table 1. The binding results for the receptor-immobilized format (Fig. 7b) are highly similar to the IgG1 Fc-immobilized format (Fig. 7a), suggesting that the extra dialysis step in the receptor-immobilized format did not affect the binding measurement significantly. Both HM-Fc and Man5-Fc displayed high affinity for FcγRIIIa with K_Ds of 26 and 33 nM, respectively, whereas the GlcNAc-Fc had lower affinity at 995 nM, and the N297Q-Fc form had no observable affinity (all measurements were conducted at 25°C). The driving factor in the difference between the high-affinity HM-Fc and Man5-Fc and the lower affinity GlcNAc-Fc was again faster dissociation of the complex (see Table 1 and supporting information at Supplemental Fig. S3). Using these methods, the SDs for determining K_D varies between the different forms, with larger overall SDs occurring for the weaker binding GlcNAc-Fc glycoform. If on the other hand, the relative SD (RSD) is considered, it is relatively consistent between all forms varying from 9.4% to 26.3% RSD with no clear indication of higher or lower RSD for weak versus strong binding glycoforms or 1 binding format versus the other. These results suggest that these 2 different binding formats are complementary in these experiments using well-defined IgG1 Fc glycoforms.

In subsequent articles in this series, these model IgG1 Fc glycoforms are used to assess how glycosylation alters the chemical stability profiles[50] and physical stability properties[49] of these glycoproteins. The physical stability data sets are then used as a model data set to develop a mathematical algorithm, based on data mining and machine learning tools, for potential use for biosimilarity assessments.[51]

In summary, we have presented here the production and biological evaluation of 4 well-defined IgG1 Fc glycoforms as the initial steps of developing a model system for biosimilarity analysis. The methods developed here allow relatively easy access to the 4 well-defined IgG1 Fc glycoforms (HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc) on a laboratory scale in quantities sufficient to enable a wide variety of biosimilarity analyses. Initial characterization of the glycoforms demonstrates that they are well-defined glycoproteins that vary significantly only in their glycosylation state, or in the case of nonglycosylated N297Q-Fc, in the presence of a conservative N to Q amino acid mutation. The 4 IgG1 Fc glycoforms form a set of sequentially truncated glycoproteins, which can be used to assess the effects of glycosylation on biological activity and protein physicochemical stability. Potential biological activity of the IgG1 Fc glycoforms was evaluated by using BLI to study the interaction of the glycoforms with the activating Fc receptor FcγRIIIa. Two BLI binding formats were developed, 1 using protein G biosensors to immobilize IgG1 Fc and 1 using C-terminally biotinylated FcγRIIIa produced by a novel method to immobilize FcγRIIIa on streptavidin biosensors. The 2 assay formats resulted in complementary information about the affinity of the IgG1 Fc glycoforms for FcγRIIIa (Table 1) and can be applied to future studies of full-length antibodies, antibody-drug conjugates, and antibodies with more complex glycosylation than the types studied here. From the perspective of developing a model system for biosimilarity analysis, the binding studies also identified members of the model system that exhibited highly similar biological activity and those with distinct differences. The HM-Fc and Man5-Fc glycoforms exhibited highly similar affinity for FcγRIIIa (approximate K_Ds of 27 and 32 nM, respectively), whereas GlcNAc-Fc had much weaker affinity (K_D of around 1000 nM), and N297Q-Fc displayed no affinity at the highest concentrations (20 μM) tested. These similarities and distinct differences in biological activity may be useful in identifying physical and chemical features that correlate with changes in biological activity, and these and other aspects of this model system will be reported in the accompanying articles in this series.[49-51] In addition, in future studies, we plan to implement additional *in vitro* enzymatic synthetic steps to produce more complex and well-defined IgG1 Fc glycoforms containing fucose and sialic acid into this model system for future biosimilarity analyses.

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