Glycoengineering of Human IgG1-Fc through Combined Yeast Expression and *in Vitro* Chemoenzymatic Glycosylation[†,§]

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ABSTRACT: The presence and precise structures of the glycans attached at the Fc domain of monoclonal antibodies play an important role in determining antibodies' effector functions such as antibody-dependent cell cytotoxicity (ADCC), complement activation, and anti-inflammatory activity. This paper describes a novel approach for glycoengineering of human IgG1-Fc that combines high-yield expression of human IgG1-Fc in yeast and subsequent *in vitro* enzymatic glycosylation, using the endoglycosidase-catalyzed transglycosylation as the key reaction. Human IgG1-Fc was first overproduced in *Pichia pastoris*. Then the heterogeneous yeast glycans were removed by Endo-H treatment to give the GlcNAc-containing IgG1-Fc as a homodimer. Finally, selected homogeneous glycans were attached to the GlcNAc-primer in the IgG1-Fc through an endoglycosidase-catalyzed transglycosylation, using sugar oxazolines as the donor substrates. It was found that the enzymatic transglycosylation was efficient with native GlcNAc-containing IgG1-Fc homodimer without the need to denature the protein, and the reaction could proceed to completion to give homogeneous glycoforms of IgG1-Fc when an excess of oligosaccharide oxazolines was used as the donor substrates. The binding of the synthetic IgG1-Fc glycoforms to the FcγIIIa receptor was also investigated. This novel glycoengineering approach should be useful for providing various homogeneous, natural or synthetic glycoforms of IgG1-Fc for structure—function relationship studies, and for future clinical applications.

Monoclonal antibodies (mAbs[1]) are an important class of therapeutic proteins and are increasingly used for the treatment of human diseases such as cancer (*1*−*4*). Human monoclonal antibodies of the immunoglobulin G (IgG) class are a multi-subunit protein complex consisting of two heavy chains and two light chains that form three domains linked together through a hinge region (Figure 1A). The three domains include two identical antigen-binding (Fab) moieties that recognize specific antigenic structures on target cells, and the Fc region that binds to various Fc receptors on effector cells to activate clearance mechanisms, such as antibody dependent cellular cytotoxicity (ADCC) and the complement cascade (*4, 5*). The IgG1-Fc region is a homodimer comprising the disulfide-linked hinge regions, the glycosylated C_H2 domains with a biantennary complex type N-glycan attached at the Asn-297, and the noncovalently associated C_H3 domains (Figure 1B). Numerous studies have demonstrated that Fc receptor-mediated effector functions of IgG antibodies are highly dependent on the patterns of Fc glycosylation (*5*). It is postulated that the Fc glycans are

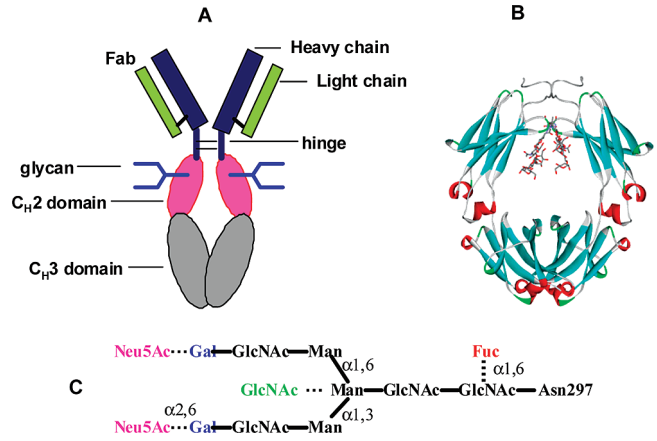

FIGURE 1: Schematic presentations of human IgG type antibody and the Fc glycans: (A) structural features of human IgG antibody; (B) a hinge-containing IgG1-Fc dimer in which the trimannose core N-glycans were remodeled at the Asn-297 sites. This model was based on the crystal structure of an anti-HIV antibody b12 (PDB code, 1hzh) (Saphire, E. O., et al. (2001) *Science 293*, 1155). (C), the structure of the N-glycans attached to Asn-297.

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[1] Abbreviations:ADCC, antibody-dependent cellular cytotoxicity; CHO, Chinese hamster ovary; ELISA, ezyme-linked immunosorbent assay; Fab, antigen-binding fragment; Fc, crystallizable (constant) fragment; FcγR, Fcγ receptor; IgG1, immunoglobulin G1; IVIG, intravenous immunoglobulin; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PNGase F, peptide:N-glycosidase F; SDS—PAGE, sodium dodecyl sulfate polyacrylamide electrophoresis; SPR, surface plasmon resonance.

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essential for maintaining a functional Fc structure required for efficient interactions of the Fc domain with Fc receptors on effector cells or the complement component C1q (*6*−*11*). Moreover, not only the presence of the N-glycans is essential for the binding of IgG1-Fc to Fcγ receptors (e.g., FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa), but the precise structure of the attached N-glycans is also critical for the affinity of the binding, and thus the effector functions of the antibodies. For example, it has been shown that core fucosylation in the Fc glycan significantly decreases Fc-mediated ADCC activity, whereas addition of a bisecting GlcNAc enhances ADCC function (*12*−*18*). There were reports that agalactosylation of the Fc N-glycans of serum IgG was associated with rheumatoid arthritis (*19*−*21*). More recently, it was shown that terminal sialylation in the Fc glycan of the intravenous IgG or its IgG-Fc fragment was essential for its anti-inflammatory activity in suppressing autoimmune diseases in animal models (*22, 23*). Therefore, the attachment of an appropriate N-glycan at the Fc domain of therapeutic monoclonal antibodies is important to maximize their therapeutic potential.

The oligosaccharide attached at the Fc region of human IgG is typically a biantennary complex type N-glycan with significant structural heterogeneity (Figure 1C). Monoclonal antibodies produced in common mammalian expression systems, e.g., CHO cells and human 293 T cells, are present as a mixture of distinct glycoforms with significant heterogeneity at the Fc glycans, from which pure glycoforms of defined structure are difficult to obtain. To enrich the glycoforms of mAbs that exhibit optimal effector functions such as enhanced ADCC, several approaches have been attempted. These include, but are not limited to, manipulations of the glycan biosynthetic pathway in the host expression system (*15, 24*−*27*). An elegant site-specific glycosylation approach through engineering a cysteine residue to replace the Asn-297 site and then ligating a synthetic oligosaccharide by an asymmetric disulfide conjugation was also reported, which resulted in IgG1-Fc analogues with a non-natural glycosidic linkage (*28*). We describe in this paper a novel approach for glycoengineering of human IgG1-Fc that combines high-yield yeast expression of human IgG1-Fc and subsequent *in vitro* chemoenzymatic glycosylation. This chemoenzymatic approach is based on the known transglycosylation activity of some endo-β-*N*-acetylglucosaminidases (ENGases), such as the Endo-A from *Arthrobactor protophormiae* (*29*−*31*) that is specific for high-mannose type N-glycans, and the Endo-M from *Mucor hiemalis* (*32*−*34*) that can act on both high-mannose and complex type N-glycans. To address the low-transglycosylation yield using natural N-glycans as the donor substrates, as well as the limit availability of natural substrates, we and other groups have explored synthetic oligosaccharide oxazolines, the mimic of the presumed oxazolinium ion intermediate generated in the substrate-assisted mechanism, as donor substrates for transglycosylation to synthesize N-glycopeptides (*35*−*42*). The use of the highly activated sugar oxazolines not only expanded the substrate availability but also dramatically enhanced the transglycosylation efficiency (*42*). The chemoenzymatic method has also shown potential for glycoprotein synthesis, as exemplified by our recent work on glycosylation remodeling of ribonuclease B (*40*). This chemoenzymatic method is particularly effective for introducing modified core N-glycans, as the ENGases (Endo-A and Endo-M) could tolerate certain modifications on the sugar oxazoline side, but the resulting glycopeptides became resistant to hydrolysis because of the slight structural modifications (*42*). Moreover, it is also possible to introduce a full-size natural N-glycan such as $Man_9GlcNAc_2$ into a GlcNAc-peptide/protein by a novel glycosynthase, EndoM-N175A mutant, that we have recently reported (*41*). EndoM-N175A can take an activated sugar oxazoline as substrate for transglycosylation, but lacks the ability to hydrolyze the resulting natural N-glycopeptide product because of the mutation. Thus EndoM-N175A represents a typical glycosynthase with novel properties as previously reported for several other glycosidases (*43, 44*). These studies implicate a great potential of the chemoenzymatic approach for synthesizing both modified and natural N-glycoproteins. We report here the expansion of this chemoenzymatic approach to glycosylation engineering of human IgG1-Fc to generate both native and synthetic homogeneous glycoforms of IgG1-Fc. Notably, we have found that the enzymatic glycosylation can be achieved on native dimeric IgG1-Fc without the need to denature the protein. In combination with the yeast expression technique, the glycoengineering described in this paper permits a quick access to an array of homogeneous glycoforms useful for structure−activity relationship studies. A preliminary investigation on the binding of these synthetic IgG1-Fc glycoforms to the FcγIIIa receptor is also described.

MATERIALS AND METHODS

Strains and Media. Escherichia coli DH5α strain was used as host for DNA manipulations. *Pichia pastoris* stain X-33 (Invitrogen, Carlsbad, CA) was used as the host for recombinant protein expression, following the manufacturer's instructions. *E. coli* was grown in TYE broth (1.5% Tryptone, 1.0% yeast extract, and 0.5% NaCl) or agar (1.5% Bacto) supplemented, as needed, with ampicillin (50 μg/mL). Yeast transformants were selected on blasticin agar (300 μg/mL blasticin). The growth medium was a buffered glycerol-complex medium (BMGY) consisting of 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol in a potassium phosphate buffer (100 mM, pH 6.0). The induction medium was a buffered methanol-complex medium (BMMY) consisting of 1.5% methanol instead of glycerol in BMGY.

Materials. The tetrasaccharide oxazoline (**3**) was prepared by chemical synthesis following our previously reported procedure (*36*); the hexasaccharide oxazoline (**5**) was synthesized according to our previously described procedure (*40*). Restriction enzymes were purchased from Promega Biosciences (San Luis Obispo, CA). Peptide N-glycosidase F (PNGase F) was obtained from New England Biolabs (Ipswich, MA). Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was overproduced in *E. coli* following the reported procedure (*45*). The pGEX-2T/Endo-A plasmid used for expressing Endo-A was kindly provided by Dr. Kaoru Takegawa. Endoglycosidase H (Endo-H) was purchased from Sigma (St. Louis, MO). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Soluble human FcγIIIa receptor was purchased from R & D Systems, Inc. (Minneapolis, MN). Protein A resin was purchased from Pierce (Rockford, IL).

Salts and buffering agents were purchased from Sigma (St. Louis, MO).

Construction of IgG1-Fc Expression Vector. A 696-base pair DNA fragment encoding human IgG1-Fc was amplified by PCR to generate *Eco*RI and *Not*I sites at 5′ or 3′ terminus respectively using the following primers: 5′-C**GA ATT C**GA GCC CAA ATC TTG TGA C-3′ (forward) and 5′-T**GC GGC CGC** TTT ACC CGG GGA CAG GGA G-3′ (reverse), where the bold-faced nucleotides indicated *Eco*RI and *Not*I sites. The PCR reaction mixture (final volume, 50 μL) contained the following: 50 ng of the template, 1 μL of primers (20 pmol), 25 μL of Premix Taq (0.05 unit/μL) containing 4 mM Mg^{2+}, and 0.4 mM dNTP. The mixture was subjected to denaturation at 95 °C for 5 min and 30 cycles at 95 °C for 10 s, 56 °C for 5 s, and 72 °C for 1 min, using a 9600 DNA Thermal Cycler (Bio-Rad, Hercules, CA). The PCR product was purified using a QIAGEN kit (QIAGEN, Valencia, CA). After digestion with *Eco*RI and *Not*I, it was cloned into the *Eco*RI and *Not*I sites of pPIC6α (Invitrogen, Carlsbad, CA) to generate the vector IgG1-Fc/pPIC6α. The whole cDNA and its flanking regions in the constructed plasmid were sequenced and confirmed to have no unexpected mutations (data not shown).

Transformation of P. pastoris. Transformation of *P. pastoris* X-33 with *Sac*I linearized expression plasmid was performed by *Pichia* EasyComp transformation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions for the expression of recombinant proteins in *P. pastoris*. After incubation for 4 days at 30 °C on YAPD agar plates containing blasticin (300 μg/mL), the recombinant colonies were selected and retained on YAPD agar plate containing blasticin (300 μg/mL) for further study.

Induction and Purification of the Recombinant Yeast IgG1-Fc. The transformants were inoculated into 10 mL of BMGY medium in a 250 mL baffled flask, and the medium was then incubated at 30 °C for 24 h with vigorous shaking. The cells, collected by centrifugation at 3000*g* for 10 min at room temperature, were transferred into 20 mL of BMMY medium in a 250 mL baffled flask, and the medium was cultivated for 96 h for induction of protein expression. Following culture, the supernatant was collected by centrifugation at 10000*g* for 10 min at 4 °C. The expression level of IgG1-Fc was measured by ELISA. For large scale preparation, the positive colonies were cultured in 500 mL of BMGY medium at 30 °C for 24 h, followed by induction culture in 1 L of BMMY medium for 96 h. The value of A_{600} at the beginning of induction was 4.5. Supernatant was collected by centrifugation at 10000*g* for 10 min at 4 °C. The recombinant IgG1-Fc was captured by affinity chromatography from the supernatant using a protein A resin. The resin was equilibrated with the binding buffer. The supernatant medium was adjusted to the same pH as that of the binding buffer, and was loaded onto the protein A column. The column was washed with 4 column volumes of binding buffer, and the recombinant IgG1-Fc was eluted with 100 mM glycine-HCl (pH 3.0). The eluted protein was neutralized immediately with 1 M Tris-HCl (pH 9.0). The pooled protein was desalted and stored at −20 °C. The yield of a typical overproduction is about 25 mg/L. The glycans attached to the IgG1-Fc were released by PNGase F treatment and were analyzed by MALDI-TOF MS.

Preparation of IgG1-Fc from Cetuximab by Papain Digestion. The IgG1-Fc fragment from mAb Cetuximab, a chimeric anti-epidermal growth factor receptor (EGFR) monoclonal antibody, was generated by papain digestion using the Antibody Fragmentation-Papain kit (PIERCE, Rockford, IL), following the manufacturer's instructions. Briefly, Cetuximab was digested into Fc and Fab fragments by papain treatment. The digest was then changed to the protein A binding buffer, and the IgG1-Fc fragment in the mixture was purified by protein A affinity chromatography.

Deglycosylation of IgG1-Fc. Complete deglycosylation of the yeast-expressed IgG1-Fc was achieved by peptide N-glycosidase F (New England BioLabs, Ipswich, MA) digestion following the manufacturer's instruction. Briefly, incubation of the recombinant IgG1-Fc (50 μg) and PNGase F (5 units, NEBL definition) in a Tris-HCl buffer (10 mM, pH8.3) at 37 °C for 4 h resulted in complete deglycosylation of the IgG1-Fc. The extent of deglycosylation was confirmed by SDS−PAGE analysis. For partial deglycosylation to prepare GlcNAc-containing IgG1-Fc (**2**), the yeast-expressed IgG1-Fc (10 mg) was treated with Endo-H (0.5 unit) in a phosphate buffer (2 mL, 50 mM, pH 6.5) at 37 °C for 10 h. SDS−PAGE indicated the completion of the deglycosylation. The GlcNAc-containing IgG1-Fc (**2**) was then purified by protein A affinity chromatography.

Size Exclusion Chromatography. The yeast expressed IgG1-Fc, the PNGase F deglycosylated IgG1-Fc and the Endo-H or Endo-A deglycosylated IgG1-Fc (500 μg in 100 μL) were each loaded on a Superdex 200 10/300 GL size-exclusion column (GE Healthcare) equilibrated with Tris-HCl (20 mM, pH 8.0) containing NaCl (150 mM). The samples were run on an AKTA FPLC system (GE Healthcare) at 4 °C with a flow rate of 0.5 mL/min. The sizes of proteins were calibrated and determined by using γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa), respectively, as standards.

Synthesis of Glycoforms of IgG1-Fc through Endo-A Catalyzed Transglycosylation. A mixture of the GlcNAc-containing IgG1-Fc (**2**) (600 μg, ca. 10 nmol for the dimer) and the tetrasaccharide oxazoline $Man_3GlcNAc$-oxazoline **3** (200 μg, 290 nmol) in a phosphate buffer (50 mM, pH 7.0, 200 μL) was incubated at 23 °C with the enzyme Endo-A (100 mU). After 3 h, SDS−PAGE indicated about 50% conversion of the IgG1-Fc into a new glycoform. Then additional oxazoline **3** (400 μg total) was added in two equal portions at 3 h intervals. After the final addition of the oxazoline, the mixture was incubated at 23 °C for 5 h when SDS−PAGE suggested the complete conversion of the IgG1-Fc to the glycoform **4**. The new IgG1-Fc glycoform (**4**) was then purified from the reaction mixture by using protein A affinity chromatography. The protein A column was equilibrated with the binding buffer. The reaction mixture, after being exchanged to the binding buffer, was loaded on a protein A column. The column was washed with 4 column volumes of binding buffer. The IgG1-Fc was then eluted with 100 mM glycine-HCl (pH 3.0). The eluted protein was neutralized immediately with 1 M Tris-HCl (pH 9.0) to give IgG1-Fc glycoform (**4**) (520 μg); the oligosaccharide attached to the IgG1-Fc was confirmed by MALDI-TOF MS analysis of the glycan released by PNGase F treatment: Calculated for $Man_3GlcNAc_2$, MW = 910.82; found (*m*/*z*), 933.50 $(M + Na)^+$. Similarly, a mixture of GlcNAc-IgG1-Fc (**2**) (300

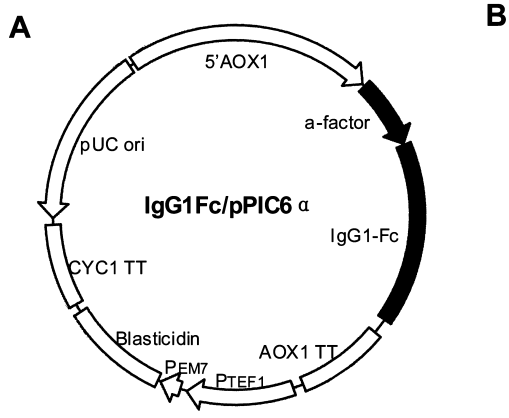

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FIGURE 2: The map of the yeast expression vector (A) and the cDNA and the amino acid sequence of the recombinant human IgG1-Fc (B). The N-glycosylation site is underscored; the *C-myc* epitope and the His-tag at the C-terminus of the recombinant protein is in bold.

μg, ca. 5 nmol for the dimer) and the hexasaccharide oxazoline **5** (300 μg, 0.295 nmol, added in 3 equal portions) in a phosphate buffer (50 mM, pH 7.0, 200 μL) was incubated in the presence of Endo-A (100 mU) for 12 h. The glycoprotein product was isolated by protein A affinity chromatography to give another glycoform of IgG1-Fc (**6**) (260 μg). The glycan attached was identified by MALDI-TOF MS analysis of the glycans released by PNGase F treatment: Calculated for $Gal_2Man_3GlcNAc_2$, MW = 1235.10; found (*m*/*z*), 1257.14 $(M + Na)^+$. The homogeneity of these synthetic glycoforms of IgG1-Fc were also assessed by SDS−PAGE under reducing conditions.

MALDI-TOF Mass Spectrometry. The glycans were released from IgG1-Fc by PNGase F treatment, using well-defined techniques (*46*). The N-glycans released were analyzed using a Bruker Autoflex II linear MALDI-TOF mass spectrometer. MALDI-TOF MS measurement was performed on an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). The instrument was calibrated by using ProteoMass Peptide MALDI-MS calibration kit (MSCAL2, Sigma/Aldirich). 2,5-Dihydroxybenzoic acid (DHB) (10 mg/mL in 50% acetonitrile containing 0.1% trifluoroacetic acid) was used as the matrix. Detailed conditions for the measurement are as follows: 337 nm nitrogen laser with 100 μJ output; laser frequency 50.0 Hz; laser power 35%; linear mode; positive polarity; detection range 800−3000; pulsed ion extraction, 70 ns; high voltage, on; realtime smooth, high; shots, 500−2000.

Surface Plasmon Resonance Measurements. The binding of FcγIIIa receptor by various glycoforms of IgG1-Fc was measured using BIAcore T100 and CM5 biosensor chips (GE Healthcare, USA) in a HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) at 25 °C. To immobilize the FcγIIIa receptor on the chips, the chip surface was activated by the injection of EDC-NHS for 7 min at 10 μL/min, followed by the injection of a solution of FcγIIIa receptor (5 μg/mL) in an acetate buffer (10 mM, pH 5.5) until the targeted 300 response units (RU) was reached. The reaction was then quenched by the addition of ethanolamine HCl (1M, pH 8.5) for 7 min at 10 μL/min. A reference cell without the ligand (FcγIIIa receptor) was prepared by a similar procedure. For affinity studies, individual Fc glycoforms at various concentrations were injected into the cells at 10 μL/min for 2 min, to ensure a steady state of binding was reached, followed by 6 min dissociation. The sensor surface was regenerated through a prolonged wash (3 min) with HBS-EP buffer until the baseline was restored. The data was then fitted into a 1:1 steady state binding model in the BIAcore T100 evaluation software and the equilibrium constant (K_D) was calculated.

RESULTS

Expression of Human IgG1-Fc in Yeast P. pastoris. Yeast *P. pastoris* was chosen as the host expression system to produce human IgG1-Fc domain. In addition to the relatively high efficiency of protein production in *P. pastoris*, hypomannosylation occurs less frequently and to a lower extent in *P. pastoris* than in *Saccharomyces cerevisiae* (*47*). The full length of human IgG1-Fc including the hinge region was cloned into the pPIC6α vector (Invitrogen) (Figure 2). The resulting recombinant plasmid was transformed into the *P. pastoris* X-33 expression strain. High-yield expression recombinant colonies were selected and used for overproduction of the IgG1-Fc under fermentation conditions. The recombinant protein was then purified from the supernatant by affinity chromatography on a protein A column. Typically 25−30 mg of recombinant IgG1-Fc was obtained from 1 L of fermentation medium. The purified IgG1-Fc appeared as a relatively broad band at ca. 60 kDa in SDS−PAGE under nonreducing conditions (without reduction) (Figure 3A, lane 2), suggesting that the IgG1-Fc exists as a homodimer in aqueous solution (phosphate buffer). This was an expected result, as the hinge-containing IgG1-Fc would form a covalently linked homodimer through disulfide-bond formation, as demonstrated in a previous report (*48*). It seems that the apparent size (60 kDa) appeared smaller than the calculated molecular weight (64−66 kDa for the dimer, based on the heterogeneously glycosylated, monomeric IgG1-Fc (estimated to be 32−33 kDa) that carries the *c-myc* epitope and the His-tag at the C-terminus. The reason was not clear, but it may be due to its compact packing for the dimer. However, the reduced IgG1-Fc appeared as a protein band at ca. 33 kDa (Figure 3A, lane 3), which was in agreement with the calculated heterogeneous glycosylated, monomeric IgG1-Fc (32−33 kDa). PNGase F treatment of the recom-

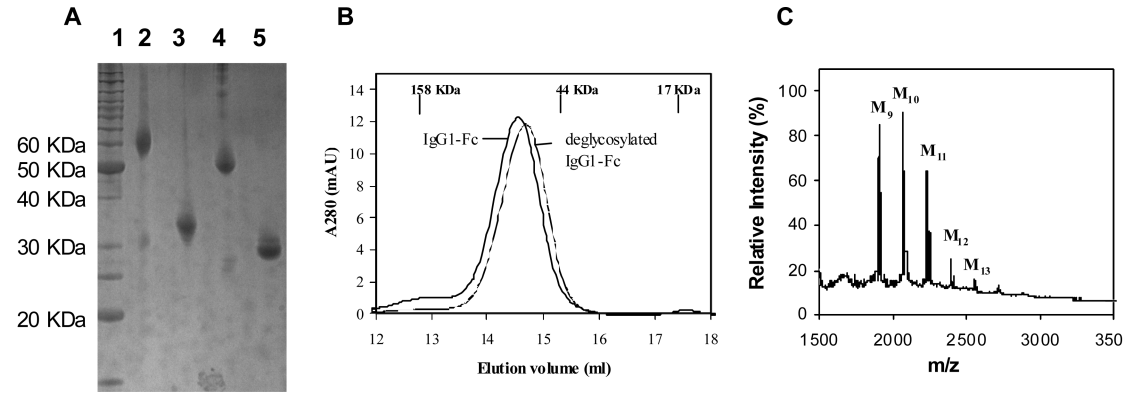

FIGURE 3: Characterization of the yeast expressed IgG1-Fc. (A) SDS−PAGE. The yeast-expressed IgG1-Fc (lanes 2 and 3) and PNGase F treated IgG1-Fc (lanes 4 and 5) are shown. SDS−PAGE were run under nonreducing conditions (lanes 2 and 4) and reducing conditions (lanes 3 and 5). Lane 1 is the protein marker. The protein bands were visualized by staining with Coomassie brilliant blue dye. (B) Gel filtration chromatography of the purified IgG1-Fc and the PNGase F-treated (deglycosylated) IgG1-Fc. The size labels (158, 44, and 17 kDa) were deduced from the appearance of the standard proteins γ-globulin, ovalbumin, and myoglobin, respectively. (C) the MALDI-TOF of the N-glycans released from the recombinant IgG1-Fc. The symbols M_9 to M_{13} represent yeast glycans $Man_9GlcNAc_2$ to $Man_{13}GlcNAc_2$, respectively.

binant IgG1-Fc resulted in the removal of the N-glycan to give protein bands that appeared at ca. 53 kDa and 31 kDa on SDS−PAGE under nonreducing and reducing conditions, respectively, implicating the deglycosylation [Figure 3A, line 4 (nonreduced form) and line 5 (reduced form)]. Again, the deglycosylated dimer (ca. 53 kDa) appeared smaller in size on SDS−PAGE than the expected molecular size (ca. 31 kDa). To verify the dimer status of the IgG1-Fc, we also performed size-exclusion chromatography (SEC) of the yeast-expressed IgG1-Fc and its deglycosylated form (Figure 3B). The deglycosylated IgG1-Fc appeared slightly smaller than the glycosylated IgG1-Fc, but both appeared as a protein larger than 50 kDa by the SEC assessment (Figure 3B). Moreover, no proteins smaller than 40 kDa were detected under the mild gel filtration conditions. These results clearly indicate that the recombinant IgG1-Fc was obtained as a homodimer. To determine the nature of the glycans attached at the Fc domain, the N-glycans in the recombinant IgG1-Fc were released by PNGase F treatment, and the glycans were analyzed by MALDI-TOF mass spectrometry. As shown in Figure 3C, the MALDI-TOF of the released N-glycans revealed the following species (*m/z*): 1905.66 (M_9), 2067.77 (M_{10}), 2229.69 (M_{11}), 2391.53 (M_{12}), and 2553.52 (M_{13}). These observed data matched well with the calculated data for the molecular ions of $[Man_9GlcNAc_2 + Na]^+$ (calculated, 1905.64), $[Man_{10}GlcNAc_2 + Na]^+$ (calculated, 2067.71), $[Man_{11}GlcNAc_2 + Na]^+$ (calculated, 2229.78), $[Man_{12}GlcNAc_2 + Na]^+$ (calculated, 2391.85), and $[Man_{13}GlcNAc_2 + Na]^+$ (calculated, 2553.92), respectively. These results suggest that the N-glycans attached in the yeast expressed IgG1-Fc are typical yeast N-glycans consisting of $Man_9GlcNAc_2$ to $Man_{13}GlcNAc_2$ species with $Man_9GlcNAc_2$, $Man_{10}GlcNAc_2$ and $Man_{11}GlcNAc_2$ as the major components.

Chemoenzymatic Glycoengineering of IgG1-Fc. The yeast expressed recombinant IgG1-Fc contains heterogeneous oligomannose-type glycans. For glycoengineering, a two-step chemoenzymatic approach was used, which involves (1) the selective deglycosylation of the yeast glycans to provide the GlcNAc-containing IgG1-Fc (**2**) and (2) attachment of a presynthesized N-glycan to the GlcNAc-primer by an endoglycosidase-catalyzed transglycosylation to form a homogeneous IgG1-Fc glycoform (**4** or **6**) (Figure 4). Treatment of the recombinant IgG1-Fc (**1**) with Endo-H, which cleaves within the chitiobiose core of the high mannose type N-glycans from glycoproteins, resulted in successful removal of the heterogeneous yeast glycans, leaving only the innermost GlcNAc attached at the Asn-297 position to give the GlcNAc-IgG1-Fc (**2**). Another enzyme, Endo-A, which is also specific for hydrolyzing high-mannose type glycans at the same site, was equally efficient in deglycosylating the yeast IgG1-Fc to produce the GlcNAc-protein (data not shown). Importantly, it was found that the Endo-H or Endo-A catalyzed deglycosylation could be achieved under native conditions in a phosphate buffer, without the need to denature the recombinant glycoprotein. The SDS−PAGE of IgG1-Fc, run after reduction, indicated that the deglycosylated product (**2**) appeared as a single band with a size of ca. 31 kDa, which was about 2 kDa less than the yeast IgG1-Fc (**1**) (Figure 5A). This result indicated that the selective deglycosylation was complete. Again, the fact that the GlcNAc-IgG1-Fc was present as a dimer after Endo-H treatment was verified by gel filtration chromatography, which showed a single protein with the size larger than 50 kDa (Figure 5B).

Endoglycosidase-catalyzed transglycosylation of the GlcNAc-containing IgG1-Fc (**2**) was first examined using $Man_3GlcNAc$-oxazoline (**3**) as the donor substrate, which is a sugar oxazoline corresponding to the trimannose core of N-glycan. The $Man_3GlcNAc$-oxazoline (**3**) was prepared by chemical synthesis following the reported procedure (*36*). The transglycosylation reaction was carried out in a phosphate buffer in the presence of Endo-A, and the reaction was monitored by SDS−PAGE. It was found that the transglycosylation reaction proceeded smoothly with the formation of a protein product that was ca. 1 kDa larger in size than the GlcNAc-protein (**2**), indicating the attachment of a glycan to the IgG1-Fc (Figure 5C, lane 3). When excess sugar oxazoline was added incrementally, a complete transglycosylation of IgG1-Fc was achieved to give the glycosylated IgG1-Fc (**4**) which appeared as a single band slightly larger than the acceptor GlcNAc-IgG1-Fc. The product was eventually isolated by protein A affinity chromatography. Similarly, when a hexasaccharyl oxazoline (**5**) was used as the donor substrate, which mimics a complex type N-glycan and was

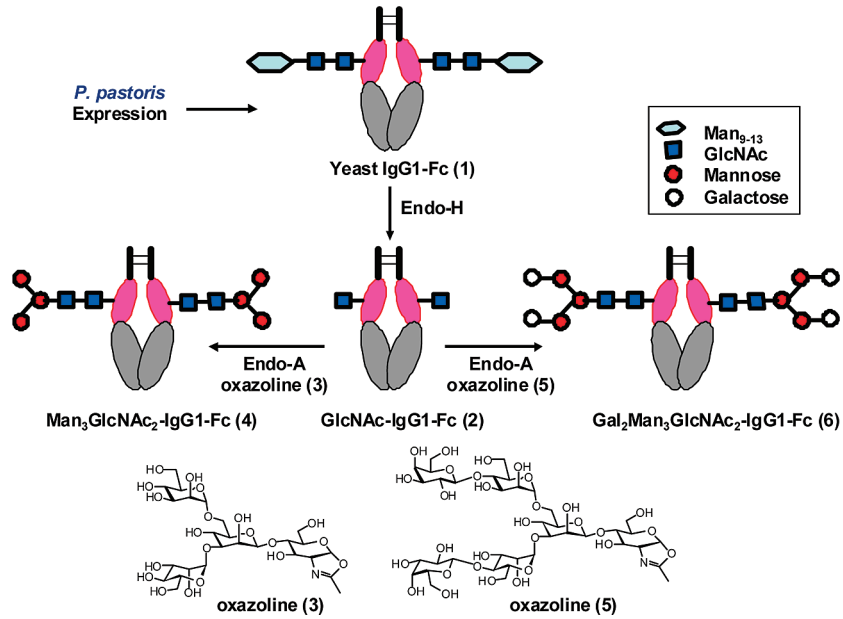

FIGURE 4: Schematic presentation of the glycoengineering of human IgG1-Fc. The heterogeneous N-glycans of the yeast-expressed IgG1-Fc were removed by Endo-H treatment. Then the GlcNAc-containing IgG1-Fc was used as an acceptor for the Endo-A catalyzed transglycosylation, with $Man_3GlcNAc$-oxazoline and $Gal_2Man_3GlcNAc$-oxazoline as the donor substrates, respectively.

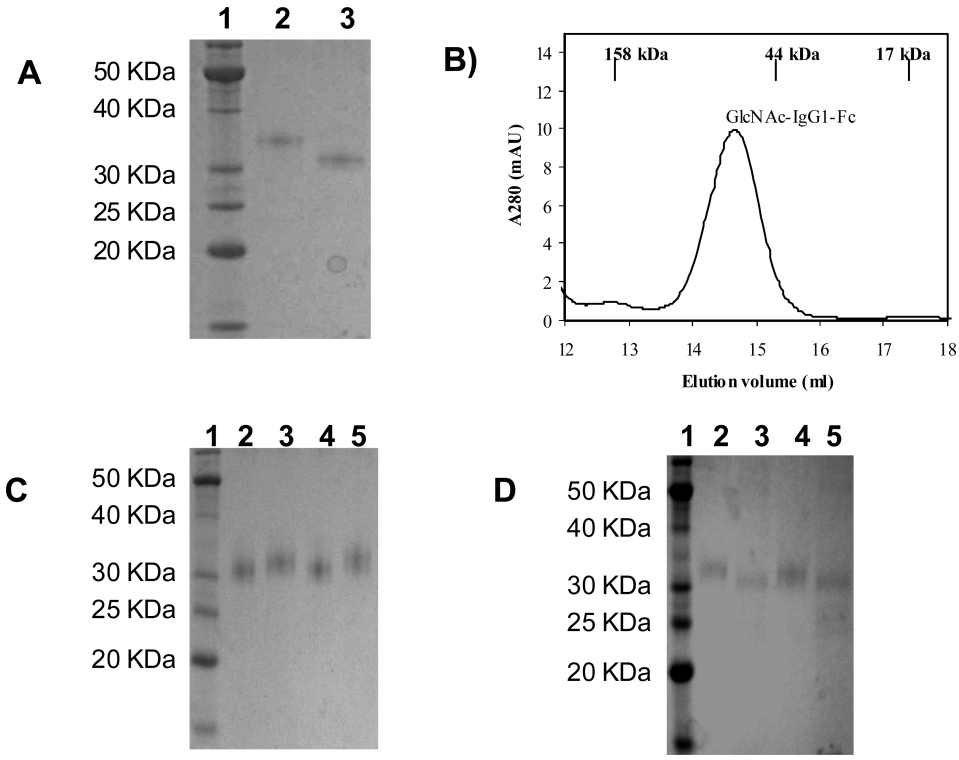

FIGURE 5: Analysis of IgG1-Fc glycoforms. (A) Deglycosylation with Endo-H: lane 1, protein marker; lane 2, recombinant IgG1-Fc (**1**); and lane 3, GlcNAc-IgG1-Fc (**2**) obtained by Endo-H treatment. (B) Gel filtration chromatography of GlcNAc-IgG1-Fc. The size labels (158, 44, and 17 kDa) were deduced from the appearance of the standard proteins γ-globulin, ovalbumin, and myoglobin, respectively. (C) Analysis of transglycosylation products: lane 1, protein marker; lane 2, GlcNAc-IgG1-Fc (**2**); lane 3, $Man_3GlcNAc_2$-glycoform (**4**); lane 4, GlcNAc-IgG1-Fc (**2**); lane 5, $Gal_2Man_3GlcNAc_2$-glycoform (**6**). (D) Analysis of transglycosylation products by PNGase F deglycosylation: lane 1, protein marker; lane 2, glycoform (**6**); lane 3, deglycosylation of **6** by PNGase F; lane 4, glycoform (**4**); lane 5, deglycosylation of **4** by PNGase F.

synthesized according to our previous report (*40*), the Endo-A catalyzed transglycosylation provided successfully another glycoform of IgG1-Fc (**6**) that carries an unnatural N-glycan $Gal_2Man_3GlcNAc_2$. The purity of the glycoengineered IgG1-Fc was confirmed by SDS−PAGE assessment (Figure 5C, lane 3 for glycoform **4** and lane 5 for glycoform **6**). It should be noted that the IgG1-Fc is a homodimer under native conditions. Therefore, the native IgG1-Fc actually contains two identical glycosylation sites at the Asn-297. When the SDS−PAGE was run under a reducing condition, only a single band from the new glycoforms (**4** and **6**) was observed, which is ca. 1 kDa and ca. 2 kDa larger in size than the GlcNAc-IgG1-Fc (**2**), respectively (Figure 5C, lane 3 for glycoform **4** and lane 5 for glycoform **6**). Although the size

difference between the glycosylated IgG1-Fc and the GlcNAc-IgG1-Fc was very small, the fact that the reduced IgG1-Fc glycoforms (**4** and **6**) appeared as a single band on SDS−PAGE implicated that both the two glycosylation sites in the new glycoforms of IgG1-Fc homodimer were occupied by the respective N-glycan. Unfortunately, an attempt to measure the molecular mass of the intact IgG1-Fc and its glycoforms either by MALDI-TOF MS or by ESI-MS all failed to provide meaningful signals, which could otherwise provide direct evidence of the purity of the glycosylated product. Nevertheless, the SDS−PAGE assessment suggested that the Endo-A catalyzed transglycosylation could efficiently attach two N-glycans to the IgG1-Fc homodimer under native conditions, without the need to denature the protein acceptor. To confirm that the transferred oligosaccharides were indeed attached to the Asn-linked GlcNAc primer on the IgG1-Fc, the two glycoengineered IgG1-Fc were treated by PNGase F, which would release N-glycans by specifically hydrolyzing the amide bond between the glycan-Asn linkage. In both cases, the deglycosylated IgG1-Fc appeared as a new single band that was ca. 1 kDa and ca. 2 kDa less than the corresponding reduced, glycosylated IgG1-Fc, respectively (Figure 5D, lanes 2 and 3 were glycoform **6** and deglycosylated **6**; lanes 4 and 5 were glycoform **4** and deglycosylated **4,** respectively). These results suggest that the N-glycans in the IgG1-Fc were attached to the GlcNAc-Asn primer in the starting GlcNAc-IgG1-Fc. Finally, the released N-glycans were subject to MALDI-TOF MS analysis (Figure 6). The MALDI-TOF MS of the glycan released from glycoform **4** gave a single species at 933.50 (*m/z*) for $[M + Na]^+$, which was in agreement with the expected glycan $Man_3GlcNAc_2$ (calculated, MW = 910.82) (Figure 6A). On the other hand, the N-glycan released from glycoform **6** showed a species at 1257.14 (*m/z*) for $(M + Na)^+$, which corresponded to the glycan $Gal_2Man_3GlcNAc_2$ (calculated MW = 1235.10) (Figure 6B). These data indicated that the glycoengineered IgG1-Fc glycoforms carried the homogeneous glycan $Man_3GlcNAc_2$ and $Gal_2Man_3GlcNAc_2$, respectively. In comparison, the N-glycans released from the IgG1-Fc of Cetuximab, a chimerized anti-EGFR monoclonal antibody, appeared as a mixture of the G0F, G1F, and G2F glycoforms (Figure 6C). The symbols G0F, G1F, and G2F are defined as the core fucosylated, biantennary complex type N-glycans released from mAbs with 0, 1, and 2 terminal galactose residues, respectively, as being commonly used for describing IgG-Fc glycoforms (*5*). The observed MS data were in good agreement with the expected mass data for the released glycans: Calculated for G0F, M = 1462.54; found, 1501.67 ($M + K^+$). Calculated for G1F, 1624.60; found, 1663.68 ($M + K^+$). Calculated for G2F, M = 1786.65; found, 1825.79 ($M + K^+$). The observed *m/z* species appeared as the potassium adduct, probably because of the presence of trace PBS that contains potassium ion. The observed heterogeneous glycoforms of Cetuximab Fc were consistent with those recently reported for Cetuximab Fc glycosylation patterns, which were identified as the fluorescent-labeled oligosaccharide derivatives (*49*).

Binding of Various IgG1-Fc Glycoforms to Fcγ*IIIa Receptor.* The activation of FcγIIIa receptor (FcγRIIIa) expressed on natural killer cells and macrophages through its interaction with IgG1-Fc of mAbs is mainly responsible for the antibody dependent cell cytotoxicity (ADCC) of

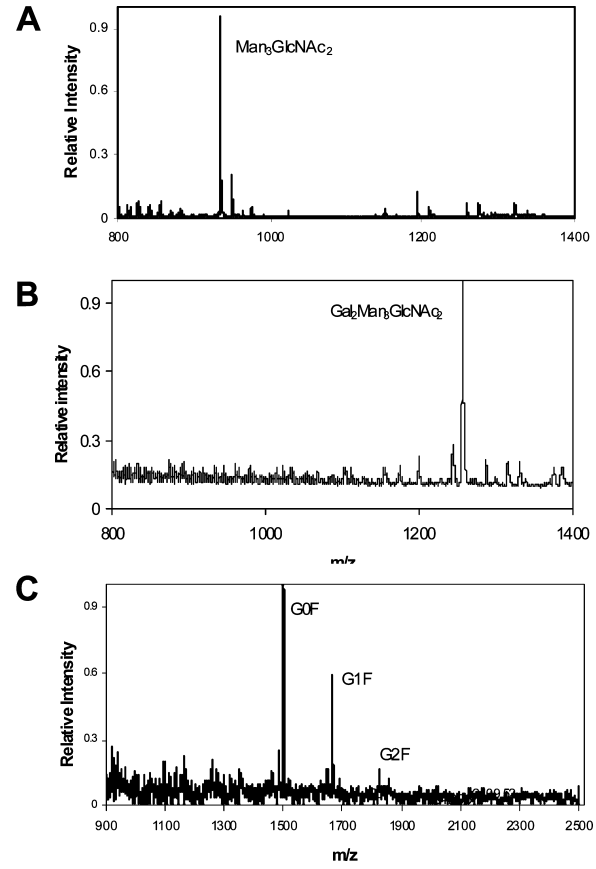

FIGURE 6: MALDI-TOF MS analysis of glycans in the IgG1-Fc glycoforms. The glycans were released from the IgG1-Fc glycoforms by PNGase F treatment and analyzed by MALDI-TOFMS. (A) Glycan released from glycoform $Man_3GlcNAc_2$-Fc (**4**). (B) Glycan released from glycoform $Gal_2Man_3GlcNAc_2$-Fc (**6**). (C) Glycans released from the IgG-Fc of Cetuximab. G0F, G1F, and G2F represent the fucosylated biantennary complex type N-glycans with 0, 1, and 2 terminal galactose residues, respectively.

therapeutic mAbs (*50*). There is cumulative evidence that the presence and the fine structure of glycans attached at the Asn-297 of IgG1-Fc region are important for the binding of Fc to FcγRIIIa (*3, 5*). In order to assess how the synthetic glycopatterns of IgG1-Fc (**4** and **6**) impact the affinity of IgG1-Fc for FcγRIIIa, we have measured the interactions of different glycoforms, including the newly prepared IgG1-Fc, with FcγRIIIa, using Biacore surface plasmon resonance (SPR) method. Recombinant human FcγRIIIa was immobilized on the chip, and its interactions with different glycoforms of IgG1-Fc were examined. Representative sensorgrams are shown in Figure 7. The K_D values were calculated by a steady-state analysis. It was observed that the apparent affinity of the yeast-expressed IgG1-Fc (**1**) carrying nonfucosylated oligomannose glycan had similar affinity as that of the CHO-expressed IgG1-Fc that bears a complex type N-glycan with core fucosylation. Interestingly, the IgG1-Fc glycoform (**4**) that carries the core pentasaccharide ($Man_3GlcNAc_2$) was 2-fold more active in binding to the FcγIIIa receptor than the CHO-expressed IgG1-Fc with core fucosylation. The IgG1-Fc glycoform (**6**) that has two galactose residues attached directly at the trimannose core was found to be about 1.4-fold more active than the CHO-expressed IgG1-Fc in binding to the FcγRIIIa. It should be pointed out that the unnatural N-glycan $Gal_2Man_3GlcNAc_2$ attached at the Asn-297 is a mimic of a typical complex type

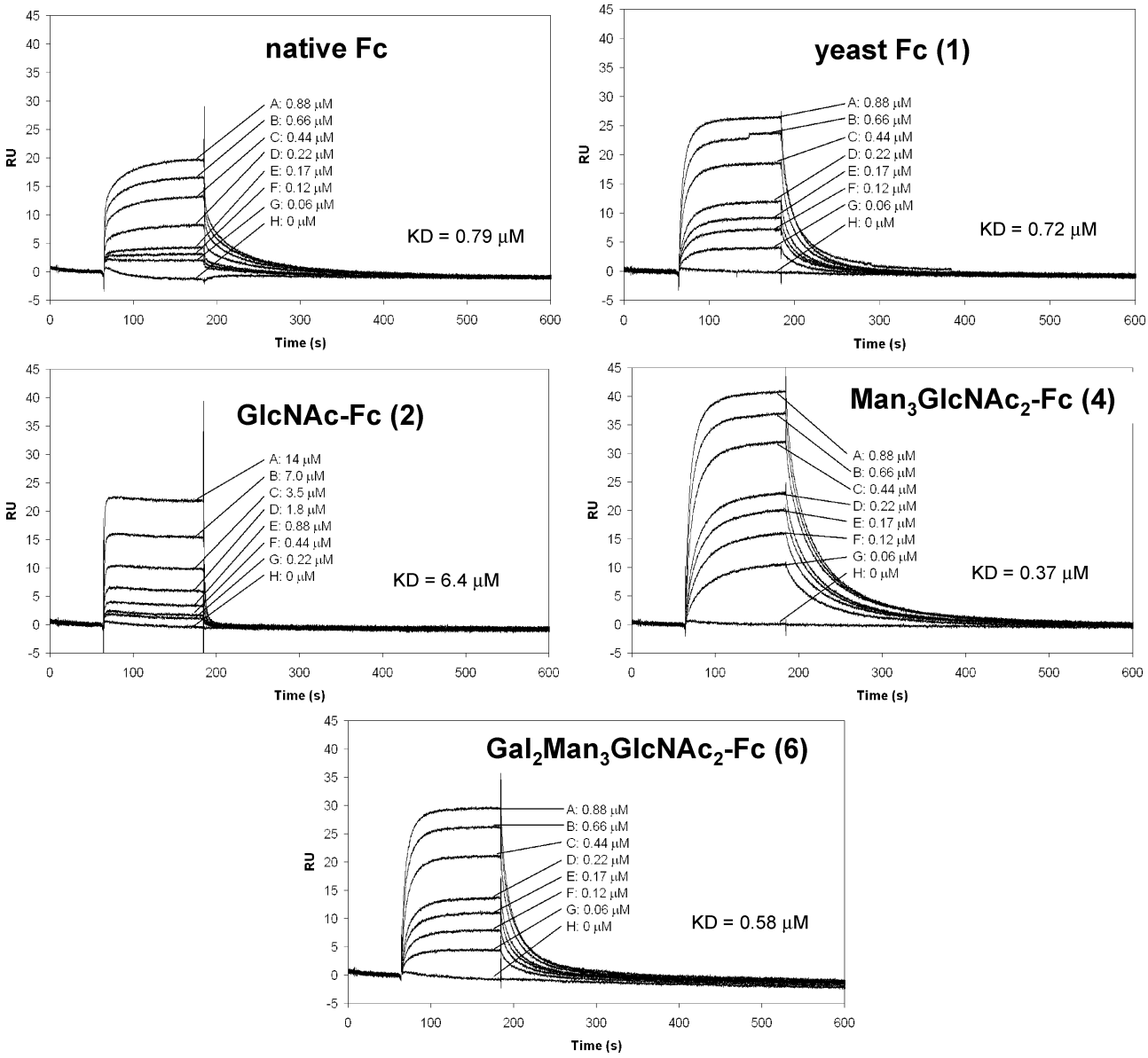

FIGURE 7: SPR sensorgrams of the binding of different IgG1-Fc glycoforms with immobilized FcγRIIIa.

N-glycan, but it misses the two internal GlcNAc moieties on the two arms. On the other hand, the glycoform GlcNAc-IgG1-Fc (**2**) that bears only the innermost GlcNAc on Asn-297 showed drastically reduced affinity, which was at least 17-fold less active than the Fc glycoform (**4**). Moreover, complete removal of the N-glycan by PNGase F treatment resulted in complete abrogation of its affinity to the FcγIIIa receptor (data not shown). These binding experiments suggested that the precise structure of the N-glycan attached at Asn-297 was crucial for a high-affinity interaction between IgG1-Fc and its host receptor FcγRIIIa that is expressed on NK cells and macrophages.

DISCUSSION

Protein glycosylation, the attachment of a carbohydrate moiety to specific amino acid residues in a protein, plays important roles in many biological recognition processes such as protein folding and quality control, cell adhesion, host−pathogen interaction, development, and immune responses (*51*−*56*). Compelling evidence has implicated that the precise structure of the attached glycans is critical for the diverse functions of glycoproteins in biological recognition processes. This is clearly true for the Fc glycosylation of IgG antibodies as related to their immune activation functions (*5, 57*). Human antibodies of the IgG class are glycoproteins that carry a conserved N-glycan at the Asn-297 of the Fc domain. Recent structure−activity relationship studies have revealed that structural variants of the attached N-glycan can differentially impact the effector functions of monoclonal antibodies, and confer upon them diverse therapeutic properties. For example, the lack of the core fucose residue and/or the presence of a bisecting GlcNAc moiety in the biantennary complex type N-glycan dramatically enhances the beneficial ADCC activity of some therapeutic mAbs (*12*−*18*). Moreover, a small fraction of the sialylated IgG glycoform in the intravenous immunoglobulin (IVIG) preparation was shown to be responsible for the anti-inflammatory activity of the IVIG, whereas the desialylated and degalactosylated glycoforms of IgG were found to be pro-inflammatory (*22, 23*). These findings underscore the importance of a precise glycosylation for the efficacy of therapeutic mAbs and/or IgG1-Fc fragments.

However, pure glycoforms of IgGs and IgG1-Fc with defined oligosaccharide structures are not easy to obtain. Monoclonal antibodies produced in mammalian expression systems, such as CHO cells and human 293 T cells, usually appear as mixtures of various glycoforms that possess the same polypeptide backbone, but differ in the composition and structure of the pendant oligosaccharides. In many cases, the active glycoforms exist only as a minor fraction that is difficult to isolate from the mixture. Several groups have devoted their efforts to enrichment of desired glycoforms of mAbs through engineering the glycan biosynthetic pathway in different host expression systems, including mammalian, plant and yeast expression systems (*15, 24*−*27*).

In the present study, we have described a novel approach for glycoengineering of human IgG1-Fc. This approach combines high-yield yeast expression of human IgG1-Fc in the well-established *Pichia pastoris* system and subsequent *in vitro* chemoenzymatic glycosylation. We have shown that the hinge-containing IgG1-Fc was overproduced in *Pichia pastoris* as a homodimer that is covalently linked by disulfide bonds. Partial deglycosylation with Endo-H (or Endo-A) and subsequent transglycosylation were performed under native conditions without the need to denature the proteins. Thus, the native structure of the IgG1-Fc homodimer was preserved during these mild transformations. Our data have shown that the enzymatic transglycosylation with synthetical sugar oxazoline as the donor substrate proceeded efficiently for the IgG1-Fc homodimer and, when excess donor substrate was used, the transglycosylation could go to completion to give a homogeneous glycoform of IgG1-Fc. A major advantage of this chemoenzymatic approach is the highly convergent enzymatic ligation of preassembled oligosaccharide moiety to the GlcNAc-IgG1-Fc that can be readily overproduced in the *Pichia* expression system. Thus the method permits totally independent manipulations of the oligosaccharide and protein portions and gives the flexibility to attach both natural and modified N-glycans at the Fc region. It should be pointed out that chemical synthesis of complex oligosaccharides often involves tedious multiple steps. Nevertheless, recent advance in synthetic method development has significantly expanded our synthetic repertoire (*58, 59*). Thus various oligosaccharide oxazolines that were required for this chemoenzymatic approach could be achievable in a good synthetic organic chemistry group on a scale sufficient for probing their structure−activity relationships (*58, 59*).

The simultaneous addition of two glycans on the native IgG1-Fc dimer through the Endo-A catalyzed transglycosylation without the need to denature the protein is of particular interest, as structural studies have suggested that the two N-glycans in IgG1-Fc homodimer are partially buried in a cleft between the two C_H2 domains, implicating that the glycans might not be well accessible (*6*−*11, 60*). Nevertheless, our experimental findings that the enzymatic transglycosylation could simultaneously attach two N-glycans to the GlcNAc-acceptor (**2**) suggest that the two GlcNAc residues in the GlcNAc-IgG1-Fc homodimer (**2**) are accessible for the Endo-A catalyzed transglycosylation. The successful preparation of the homogeneous glycoforms (**4** and **6**) suggests that this combined glycoengineering approach would be feasible for preparation of various defined glycoforms of IgG1-Fc for structure−activity relationship studies and for potential clinical applications.

Antibody-dependent cellular cytotoxicity (ADCC) is a major effector function that monoclonal antibodies can trigger for achieving their therapeutic benefit. Many studies have shown that not only is ADCC dependent on the presence of the Fc glycosylation, but the efficiency of cell-mediated killing is sensitive to the precise structure of the glycans attached at the Fc domain (*12*−*18, 26, 27*). Moreover, ample evidence has indicated that high-affinity binding of IgG1-Fc for FcγIIIa receptor on natural killer (NK) cells correlates to the enhanced ADCC of human antibodies. In this study, we have assessed the binding of human FcγIIIa receptor to different glycoforms of IgG1-Fc thus obtained. Our results have shown that the glycoform (**4**) carrying the core N-pentasaccharide was 2-fold more active in binding to human FcγIIIa receptor than the Cetuximab-derived IgG1-Fc, which carries core fucosylated N-glycans terminated with 0−2 galactose residues. The glycoform (**6**) that bears an unnatural complex glycan mimic showed 1.4-fold increase in affinity to FcγIIIa receptor than the Cetuximab-derived IgG1-Fc. In contrast, the partially deglycosylated IgG1-Fc, GlcNAc-IgG1-Fc (**2**), was at least 17-fold less active than the glycoform (**4**) in binding to FcγIIIa receptor. Our experimental data clearly show that indeed the glycan structure at the Fc domain provides a fine-tuning for the Fc's affinity to its receptor.

Identification of novel, high-affinity glycoforms of antibodies is clinically important for maximizing the efficacy of therapeutic mAbs. It was found that there were polymorphic variants in patients for some Fcγ receptors, such as FcγRIIa and FcγRIIIa on macrophage and NK cells which demonstrate significantly different affinity to mAbs (*61*). In human FcγRIIIa, for example, two variants exist in which the amino acid residue at the 158 position is either a valine (V158) or a phenylalanine (F158) as a result of point mutation. It has been demonstrated that the FcγRIIIa-F158 variant has a much lower affinity for IgG1-Fc. This was reflected by the fact that NK cells expressing the FcγRIIIa-F158 exhibited much less NK cell-mediated killing than the V158 variant (*62*). A recent clinical study on Rituximab (an anti-CD20 mAb) for the treatment of non-Hodgkin's lymphoma has made a clear correlation between the patients' therapeutic responses and their FcγRIIIa-V/F polymorphisms (*63*). The clinical results have indicated that patients with the high-affinity FcγRIIIa-V158 polymorphism responded to Rituximab much better than those with the lower affinity FcγRIIIa-F158 polymorphism (*63*). Since the commercially available mAbs such as Rituximab are less effective for the patients with the FcγRIIIa-F158 polymorphism, a useful approach is to engineer the antibody's Fc glycosylation to create high-affinity glycoforms for the FcγRIIIa-F158. The chemoenzymatic approach to glycosylation engineering of IgG1-Fc described here provides a valuable means to achieve this purpose.

In summary, an efficient glycoengineering approach was established that combines the power of recombinant DNA technology (yeast expression) and *in vitro* chemoenzymatic synthesis. This convergent glycoengineering approach is flexible for synthesizing both natural and unnatural glycoforms of IgG1-Fc, which will be useful for structure−activity relationship studies for identifying novel glycoforms with

potent efficacy for future clinical applications. The extension of this approach to the synthesis of various new, homogeneous glycoforms of IgG1-Fc and to the glycoengineering of full-size mAbs is in progress in our laboratory, which will be reported in due course.

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