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Chemoenzymatic Glycoengineering of Intact IgG Antibodies for Gain of Functions

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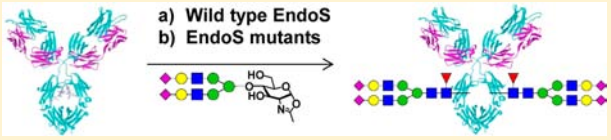
Supporting Information

ABSTRACT: The fine structures of Fc *N*-glycans can modulate the effector functions of IgG antibodies. It has been demonstrated that lack of the core fucose on the Fc *N*-glycans leads to drastic enhancement of antibody-dependent cellular cytotoxicity (ADCC), while terminal α2,6-sialylation of Fc glycan plays a critical role for the anti-inflammatory activity of human intravenous immunoglobulin (IVIG). We describe in this paper a highly efficient chemoenzymatic method for site-selective Fc glycoengineering of intact monoclonal antibody and IVIG. Two new glycosynthase mutants (EndoS-D233A and D233Q) were generated by site-directed mutagenesis of EndoS (an endoglycosidase from *Streptococcus pyogenes*) and were found to be capable of efficiently transferring predefined *N*-glycans from corresponding glycan oxazolines to the Fc-deglycosylated intact IgGs without product hydrolysis. As a model study, rituximab (a therapeutic monoclonal antibody) was successfully transformed from mixtures of G0F, G1F, and G2F glycoforms to well-defined homogeneous glycoforms, including a fully sialylated (S2G2F) glycoform that may gain anti-inflammatory activity, a nonfucosylated G2 glycoform that showed significantly enhanced FcγIIIa receptor-binding activity, and an azido-tagged glycoform that can be further transformed into other glycoforms. We also found that EndoS could selectively remove the Fc *N*-glycans in the presence of FAB glycosylation. This finding, coupled with the remarkable transglycosylation activity of the EndoS glycosynthase mutants, permitted a highly selective glycoengineering of the IVIG's Fc glycans into a fully sialylated Fc glycoform, which may possess significantly enhanced anti-inflammatory activity. The glycoengineering approach described here provides a general platform to modulate the effector functions of IgG antibodies, enabling the optimization of therapeutic efficacy and gain of new functions of monoclonal antibodies and IVIG.



INTRODUCTION

Monoclonal antibodies (mAbs) of the IgG type are an important class of therapeutic proteins used for the treatment of cancer, autoimmune, and infectious diseases.[1−3] IgG antibodies are composed of two heavy chains and two light chains that are associated to form three distinct protein domains, including two variable Fab domains and a constant (crystallizable) Fc domain linked by a flexible hinge region. The Fab domains are responsible for antigen binding, while the Fc domain is engaged in Fc receptor-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).[2,4] The Fc domain is a homodimer bearing two *N*-glycans at the conserved *N*-glycosylation sites (N297). The attached oligosaccharides are biantennary complex type with considerable structural heterogeneity, in which the *N*-linked heptasaccharide core can be differentially decorated with core fucose (Fuc), bisecting *N*-acetylglucosamine (GlcNAc), terminal galactose (Gal), and terminal sialic acid (Sia) (Figure 1).[5−7] X-ray crystallographic and NMR structural studies indicate that the Fc glycans are sandwiched between the two C_H2/C_H3 subdomains and have multiple noncovalent interactions with the Fc domains.[8−14] These studies have shown that the attachment of different Fc glycans can have distinct impact on the Fc domain conformations, implicating an important role of glycosylation in maintaining an appropriate Fc domain structures for interactions with respective Fc receptors associated with antibody's effector functions.[8−14]

It has been further demonstrated that the fine structures of Fc *N*-glycans are important determinants of the pro- and anti-inflammatory activities of antibodies.[2,15] For example, the lack of the core fucose, as well as the attachment of a bisecting GlcNAc moiety, dramatically enhances the affinity of antibody for the FcγIIIa receptor (FcγRIIIa), which is responsible for the antibody-dependent cellular cytotoxicity (ADCC).[11,16−18] Thus, low-fucose content mAbs are sought out for improved in vivo anticancer efficacy.[19,20] On the other hand, the terminal α-2,6-sialylated Fc glycoform, a minor component of the intravenous immunoglobulin (IVIG) pooled from the sera of thousands of healthy blood donors, was recently identified as the active species for the anti-inflammatory activity of IVIG in a mouse model of rheumatoid arthritis (RA).[21−23] However, commercially available IgGs, including monoclonal antibodies and IVIG, typically exist as mixtures of glycoforms that are not optimal for their respective therapeutic activities. For instance, the major Fc glycoforms of monoclonal antibodies currently used for cancer treatment are core-fucosylated that possess

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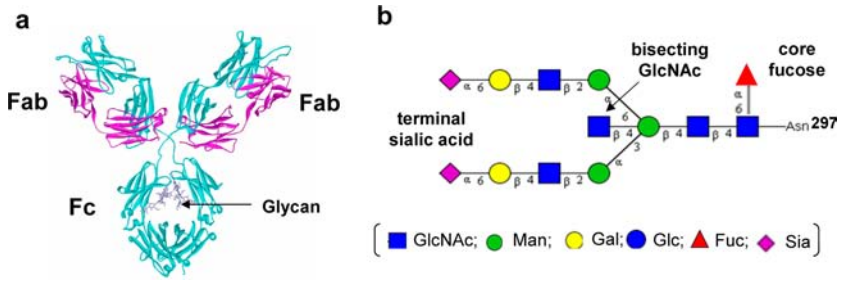

Figure 1. Structures of a typical IgG antibody and the Fc *N*-glycans. (a) Alpha backbone structure of a human IgG showing its functional regions (modeled on the basis of PDB code 1HZH): purple, light chain; cyan, heavy chain; navy blue, Fc glycans. (b) The structure of a full-length biantennary complex type *N*-glycan attached to the Asn297 in the Fc domain.

relatively low affinity for the activation receptor FcγRIIIa, demonstrating low efficacy particularly for those patients with the low-affinity FcγRIIIa-F158 allelic polymorphism.[2,19,20]

The impact of glycosylation on the biological functions and therapeutic outcome of IgG antibodies has stimulated tremendous interest in developing methods to control antibody's glycosylation. One approach is to control the glycosylation profiles during production through glycan biosynthetic pathway engineering in various expression systems, including mammalian, plant, and yeast host cells.[24−30] This has resulted in the production of low-fucose or nonfucosylated monoclonal antibodies with improved ADCC activities. But, the glycoforms that can be generated by this approach have been limited, and in most cases, a complete control to a defined homogeneous glycoform is difficult. A recent analysis of several therapeutic glycoprotein drugs on the market, including monoclonal antibody rituximab, has indicated significant changes of the glycosylation profiles from different batches produced in different periods.[31] This analysis implicates the challenge in maintaining consistent production of glycoprotein-based drugs and also raises regulatory concerns, as changes of the Fc glycosylation would most likely impact the therapeutic efficacy. An alternative approach to addressing the inconsistence and heterogeneity in glycosylation of glycoproteins is to perform glycosylation remodeling through trimming off the heterogeneous *N*-glycans and extending the sugar chains by enzymatic glycosylation.[32,33] We have recently described a chemoenzymatic method for Fc glycosylation remodeling taking advantage of the transglycosylation activity of several endoglycosidases and their glycosynthase mutants using glycan oxazolines as their substrates.[34−36] This approach consists of two steps: trimming off all the heterogeneous *N*-glycans by an endoglycosidase to leave only the first GlcNAc at the glycosylation site(s) and then adding back a well-defined *N*-glycan en bloc via an endoglycosidase-catalyzed transglycosylation reaction.[32] Our recent work has demonstrated that IgG-Fc domain glycosylation engineering can be achieved by a combination of yeast or CHO cell expression of the Fc domain and its subsequent chemoenzymatic remodeling through an enzymatic deglycosylation/reglycosylation approach.[34−36] We have shown that the endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*, EndoA, is highly efficient to glycosylate the GlcNAc-containing Fc domain by using various synthetic *N*-glycan core oxazolines as substrates.[34,35] Nevertheless, the limitations of the current status of the method are apparent: (a) neither EndoA nor EndoM (another endoglycosidase from *Mucor hiemalis*) was able to transform core-fucosylated IgG-Fc domain,[35] the major glycoforms of recombinant mAbs and IVIG; (b) EndoD mutants were able to attach a Man3GlcNAc core to a fucosylated GlcNAc-Fc domain,[36] but none of EndoD, EndoA, EndoM, and their mutants[36−39] were capable of transferring intact complex type *N*-glycan to either fucosylated or nonfucosylated GlcNAc-Fc domain; and (c) glycosylation remodeling of intact full-length IgG antibodies with complex type *N*-glycans is yet to be achieved. As part of our program aiming to develop efficient enzymatic deglycosylation/glycosylation system for glycoprotein glycosylation remodeling, we turned our attention to EndoS, an endo-β-N-acetylglucosaminidase (ENGase) from *Streptococcus pyogenes* that is capable of hydrolyzing the Fc *N*-glycans of intact IgG antibodies by cleaving the β-1,4-glycosidic bond in the chitobiose core of the *N*-glycans.[40−42] In this paper, we report two novel glycosynthase mutants (EndoS-D233A and EndoS-D233Q) generated by site-directed mutagenesis, which showed remarkable transglycosylation efficiency capable of transferring complex type *N*-glycans from activated glycan oxazolines to deglycosylated intact antibodies without product hydrolysis. We found that the two mutants acted efficiently on both core-fucosylated and nonfucosylated GlcNAc-Fc domain of intact antibodies to provide various defined IgG glycoforms. As selected examples, monoclonal antibody rituximab was transformed from a mixture of G0F, G1F, and G2F glycoforms to a Fc fully sialylated glycoform (S2G2F) that is expected to gain an anti-inflammatory activity, a homogeneous nonfucosylated (G2) glycoform that would have optimal ADCC activity, and an azido-tagged rituximab glycoform that could be further functionalized through orthogonal ligation. In addition, commercial IVIG that contains less than 10% of Fc sialylated glycoforms was transformed to an IVIG preparation carrying >90% Fc sialylated glycoforms. In this case, a remarkable selectivity on Fc glycoengineering was achieved without altering the FAB glycosylation patterns in IVIG.

■ RESULTS AND DISCUSSION

Generation of EndoS Glycosynthase Mutants and Their Use for Glycosylation Remodeling of Intact Monoclonal Antibody Rituximab. We have previously created glycosynthases from several GH85 endoglycosidases (ENGases), including EndoA, EndoM, and EndoD, by site-directed mutagenesis of a key asparagine (Asn) residue responsible for promoting oxazolinium ion intermediate formation during hydrolysis.[36−39,43] EndoS is an endoglycosidase belonging to the glycoside hydrolase family 18 (GH18),[40,41] which is in the same GH family as EndoF1, EndoF2, and EndoF3 that were recently shown to have transglycosylation activity.[44] Based on the assumption that EndoS-catalyzed hydrolysis also proceeds by a substrate-assisted mechanism involving the formation of an oxazolinium

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EndoS    181 RTIPWRFLAGGDNSGIAEDTSKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVDVEHDSIP 240
EndoF3   123 QNIDDDVSWQSSKPGGFASAAAYG----------DAIKSIVIDKWKLDGISLDIEHS--- 169
             :.*   .   ..:.*   .:: *           .** .  : *::***:.:*:**.
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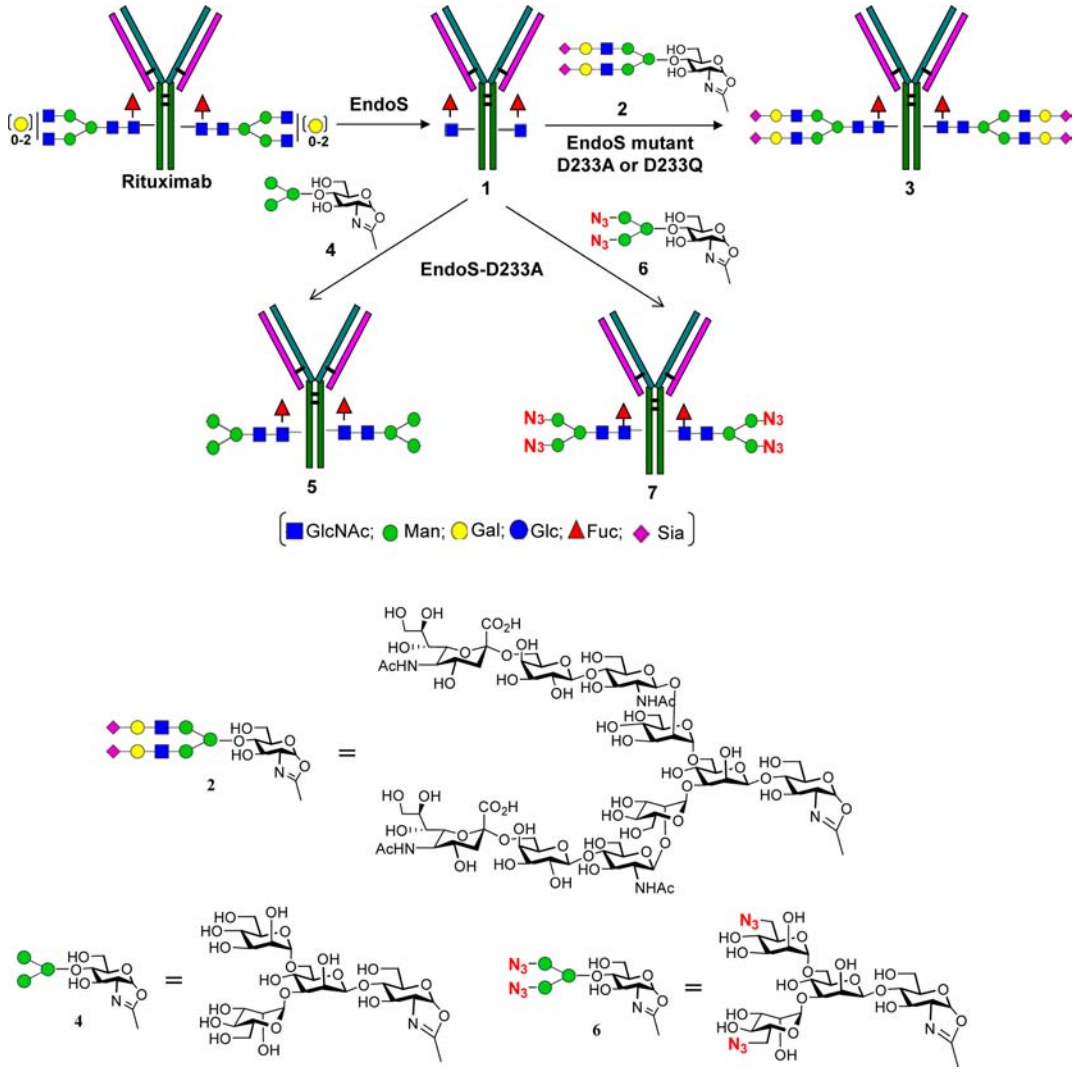
Figure 2. Sequence alignment of EndoS and EndoF3.

Scheme 1. Glycosylation Remodeling of Rituximab To Prepare Homogeneous Natural and Selectively Modified Glycoforms



ion intermediate, as demonstrated by other GH18 endoglycosidases such as EndoF3,[45] we sought to create potential glycosynthases from EndoS by identifying and mutating the residue responsible for promoting oxazolinium ion formation. Previous structural and mutagenesis studies on EndoF3 have shown that an aspartic acid residue at position 165 (D165), instead of an asparagine residue as in the family GH85 enzymes, is responsible for promoting oxazoline formation and that the E167 residue is the general acid/base for catalytic hydrolysis.[45] Sequence alignment of EndoS with EndoF3 led to the identification of two key residues in EndoS for catalysis: the D233 residue (corresponding to D165 in EndoF3) responsible for promoting oxazolinium ion formation and the E235 residue (equivalent to E167 of EndoF3) as the general acid/base residue in glycan hydrolysis (Figure 2). Functionally, the D233 residue should be also equivalent to the N171, N175, and N322 in the GH85 endoglycosidases, EndoA, EndoM, and EndoD, respectively. Thus, following the approach to creating glycosynthases from EndoA, EndoM, and EndoD that proceed in a substrate-assisted mechanism via an oxazolinium ion intermediate,[36−39] we generated two specific mutants, D233A and D233Q by site-directed mutagenesis of EndoS. These mutants, as well as the wild-type EndoS, were expressed in *Escherichia coli* in high yield (30−40 mg/L) as a GST fusion protein and purified by glutathione affinity chromatography.

Rituximab, a therapeutic monoclonal antibody, was used as a model mAb to examine the deglycosylation activity and potential transglycosylation activity of the enzymes. The major Fc glycans of commercial rituximab are core-fucosylated biantennary complex type oligosaccharides carrying 0−2 galactose moieties named G0F, G1F, and G2F glycoforms, respectively, as revealed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of the *N*-glycans released by PNGase F (Figure S1, Supporting Information). Treatment of rituximab with the EndoS−GST fusion protein (here, referred as wild-type EndoS

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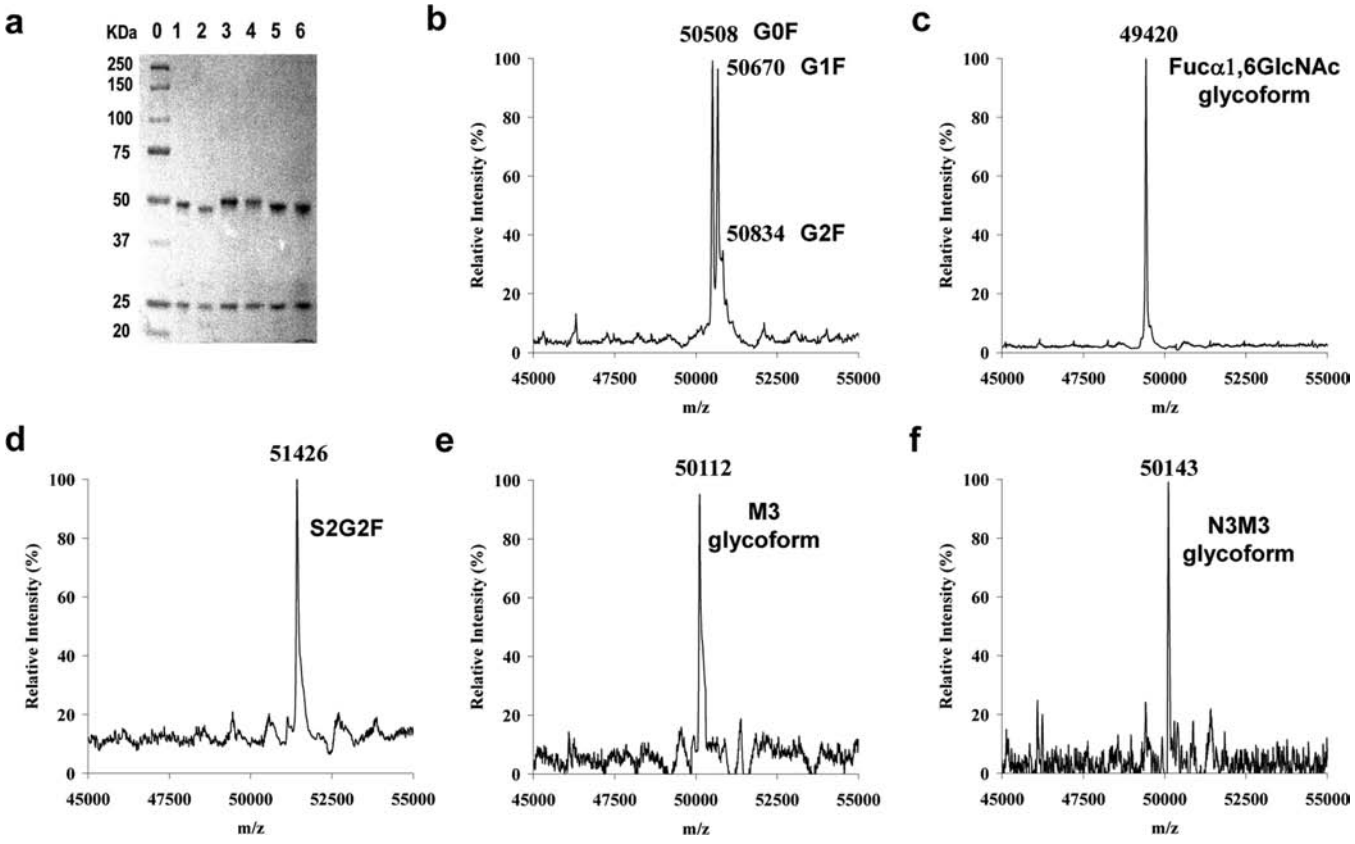

Figure 3. SDS-PAGE and ESI-MS analysis of the glycosylation remodeling of rituximab. (a) SDS-PAGE analysis: lane 0, protein markers; lane 1, commercial rituximab; lane 2, EndoS deglycosylated rituximab (**1**); lane 3, transglycosylation product (**3**) from the EndoS-D233A catalyzed reaction between **1** and sialoglycan oxazoline (**2**); lane 4, transglycosylation product from the EndoS-D233Q catalyzed reaction of **1** and **2**; lane 5, the transglycosylation product (**5**) from the EndoS-D233Q catalyzed reaction between the deglycosylated rituximab (**1**) and Man3GlcNAc oxazoline (**4**); lane 6, the transglycosylation product (**7**) from the EndoS-D233Q catalyzed reaction between the deglycosylated rituximab (**1**) and N3Man3GlcNAc oxazoline (**6**). (b) ESI-MS (after deconvolution) of the heavy chain of the commercial rituximab. (c) ESI-MS of the deglycosylated rituximab (**1**). (d) ESI-MS of the transglycosylation product (**3**). (e) ESI-MS of the transglycosylation product (**5**). (f) ESI-MS of the transglycosylation product (**7**).

or EndoS) resulted in a rapid deglycosylation to give the corresponding Fc *N*-glycans (with only one GlcNAc at the reducing end) (Figure S2, Supporting Information) and the deglycosylated rituximab that bears the fucosylated GlcNAc disaccharide moiety (Fucα1,6GlcNAc) at the glycosylation sites (N297). These results confirm the remarkable Fc glycan-hydrolyzing activity of the wild-type EndoS on intact IgG, implicating its usefulness in the first step for glycosylation remodeling of mAbs. The transglycosylation potential of EndoS and its mutants was then examined using the deglycosylated rituximab as the acceptor and several synthetic glycan oxazolines as the donor substrates, as depicted in Scheme 1. The glycosylation remodeling process was monitored by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography mass spectrometry (LC-MS) analysis (Figure 3). The heavy chain and light chain of rituximab appeared at approximately 50 KDa and approximately 25 KDa, respectively, under reducing conditions (Figure 3a, lane 1). After deglycosylation with wild-type EndoS, the heavy chain appeared as a single band at approximately 48 KDa, suggesting the removal of the two *N*-glycans (each from a heavy chain) in rituximab (Figure 3a, lane 2). Incubation of the deglycosylated rituximab (**1**) and the synthetic sialoglycan oxazoline (**2**)[46] (donor/acceptor, 50:1, molar ratio) with mutant EndoS-D233A gave a transglycosylation product (**3**), the heavy chain of which appeared as a single band that was about 2 KDa larger than that of the deglycosylated rituximab (**1**) (Figure 3a, lane 3). This result suggests that a new *N*-glycan was attached to each of the Fc heavy chains. Incubation of **1** and **2** with EndoS-D233Q gave the same transglycosylation product (Figure 3a, lane 4). Interestingly, an essentially quantitative transglycosylation for the Fc domain of the intact antibody was achieved within 1 h incubation. It was found that a longer incubation (10 h) did not lead to hydrolysis of the transglycosylation product. These results indicate that the two EndoS mutants are new efficient glycosynthases that enable the glycosylation of deglycosylated intact IgG with complex type *N*-glycan without product hydrolysis.

We further characterized the transglycosylation by LC-MS analysis. The heavy chain and light chain of rituximab were separated under a LC-MS condition (Figure S3, Supporting Information). Deconvolution of the light chain MS data gave a mass of 23 044, which was consistent with the calculated mass of rituximab light chain (M = 23 042 Da).[47] Deconvolution of the MS data of the heavy chain gave three distinct m/z species, 50508, 50670, and 50834 (Figure 3b), which were in good agreement with the theoretical mass of heavy chain glycoforms: G0F, M = 50 515 Da; G1F, M = 50 677 Da; and G2F, M = 50 839 Da; respectively.[47] The deconvoluted electron spray ionization mass spectrometry (ESI-MS) of the heavy chain of

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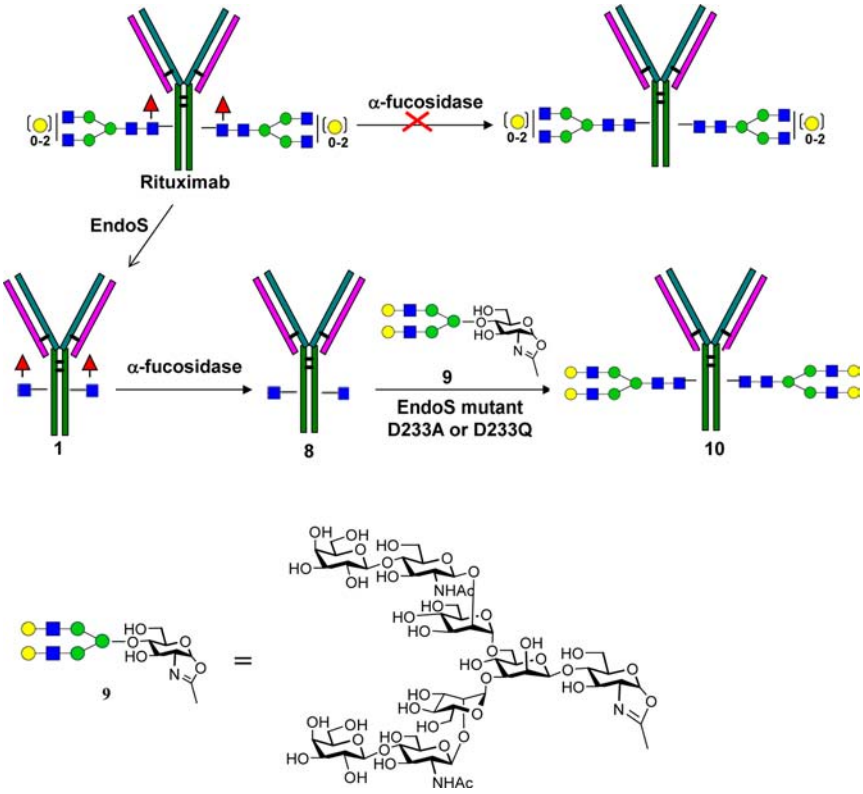
Scheme 2. Enzymatic Remodeling to Nonfucosylated Homogeneous Glycoform of Rituximab



the deglycosylated rituximab (**1**) showed a single species at 49 420 (Figure 3c), which matched well with a heavy chain carrying a Fucα1,6GlcNAc disaccharide moiety (calculated, M = 49 420 Da). After glycosylation remodeling, a single peak at 51 426 was observed from the heavy chain of the transglycosylation product (**3**), with an addition of 2006 Da to the deglycosylated heavy chain of the rituximab (Figure 3d). This result indicates the attachment of a sialoglycan from the corresponding sugar oxazoline (**2**) to the heavy chain. The single band on SDS-PAGE and the neat MS spectra of the transglycosylation product clearly suggests that the transglycosylation was essentially quantitative on the two glycosylation sites of the Fc domain in rituximab (incomplete glycosylation of any of the two sites in the Fc homodimer would result in the observation of the Fucα1,6GlcNAc-heavy chain after reduction, M = 49 420 Da). To further confirm that the *N*-glycan was specifically attached to the GlcNAc of the Fc domain, we released the whole *N*-glycan from the glycoremodeled rituximab (**3**) by treatment with PNGase F, which specifically hydrolyzes the amide bond between the Asn−glycan linkage. The released *N*-glycans were labeled by fluorescent tag 2-aminobenzamide (2-AB) and were subjected to fluorescent high-performance liquid chromatography (HPLC) and MS analysis. The LC-MS analysis clearly revealed that the released *N*-glycan was the expected biantennary complex type *N*-glycan carrying core fucose and terminal sialic acids, which consisted of approximately 92% disialylated *N*-glycan and approximately 8% monosialylated *N*-glycan (Figure S4b, Supporting Information). The *N*-glycan composition was well consistent with the ratio found in the corresponding *N*-glycan oxazoline (**2**) used for the transglycosylation. This result confirms that the transferred *N*-glycan was specifically attached to the GlcNAc primer in the deglycosylated rituximab. This study represents the first report of glycosylation remodeling of an intact IgG monoclonal antibody with an en bloc transfer of a full-size natural complex type *N*-glycan to the Fc domain through a highly efficient deglycosylation−reglycosylation protocol enabled by the combined use of EndoS and EndoS-based glycosynthase. After completion of the transglycosylation, the product was purified by a simple protein A affinity chromatography, giving the well-defined homogeneous glycoform. It should be pointed out that the commercial rituximab contains only trace amount of sialylated glycoform (Figure S4a, Supporting Information). Since sialylated Fc and IgG were proposed to have anti-inflammatory activity, the glycoengineered rituximab carrying fully sialylated Fc *N*-glycans may gain an anti-inflammatory function, thus potentially expanding its therapeutic coverage from cancer treatment to the treatment of autoimmune diseases.[21,22]

In addition to the sialylated complex type *N*-glycan oxazoline (**2**), the EndoS mutants were equally efficient to use the Man3GlcNAc core oxazoline (**4**)[48] and the azido-tagged N3Man3GlcNAc oxazoline (**6**)[49] for rituximab glycoengineering, leading to the formation of the corresponding homogeneous glycoforms, **5** and **7**, respectively (Scheme 1). The deconvoluted ESI-MS of the heavy chain of the transglycosylation product (**5**) showed a single species at 50 112 (Figure 3e), which matched well with the calculated molecular mass (M = 50 109 Da) of the rituximab heavy chain carrying a Man3GlcNAc2 glycan. Similarly, the deconvoluted ESI-MS of the heavy chain of transglycosylation product 7 showed a single species at 50 143 (Figure 3f), which was in good agreement with the calculated molecular mass (M = 50 134 Da) of the rituximab heavy chain carrying a N3Man3GlcNAc2 glycan. Again, these results indicate that the transglycosylation is essentially quantitative. It should be mentioned that decreasing

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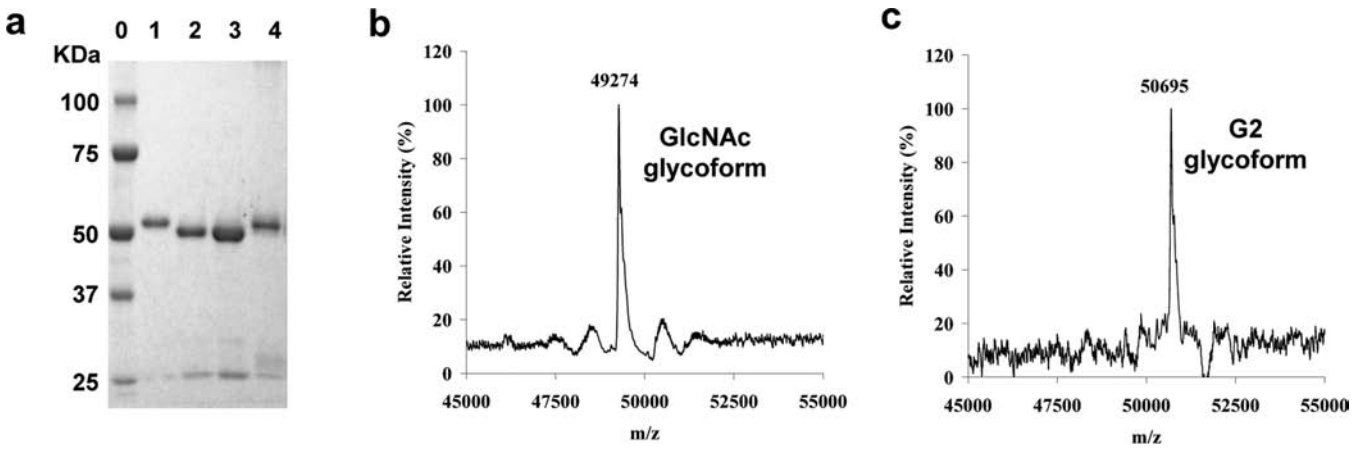

Figure 4. SDS-PAGE and ESI-MS analysis of glycoengineering of rituximab to the nonfucosylated G2 glycoform. (a) SDS-PAGE analysis: lane 0, protein markers; lane 1, commercial rituximab; lane 2, the EndoS deglycosylated rituximab (**1**); lane 3, the defucosylated product (**8**); lane 4, the glycoengineered G2 glycoform. (b) ESI-MS (after deconvolution) of the heavy chain of the defucosylated rituximab (**8**). (c) ESI-MS of the heavy chain of the glycoengineered G2 rituximab (**10**).

the molar ratio of donor/acceptor to 25:1 still resulted in efficient transformation, implicating the remarkable transglycosylation efficiency of the EndoS glycosynthase mutants. In particular, the selective introduction of azide functionality on the core of the Fc *N*-glycan in intact monoclonal antibodies will allow further site-specific modifications of antibodies through click chemistry,[50,51] which may be explored for labeling and targeting purposes, or for expanding the diversity of antibody glycoforms for further structure−activity relationship studies.

We also tested the wild-type EndoS for transglycosylation of deglycosylate rituximab (**1**) with the glycan oxazolines **2** and **4** under the same conditions as we did for the EndoS mutants; we observed only transient formation of the corresponding transglycosylation products as monitored by LC-MS, probably due to quick in situ hydrolysis of the products by the wild-type enzyme. Recently, Scanlan, Davis, and co-workers reported an independent study on the substrate specificity of EndoS and demonstrated that wild-type EndoS could use Man3GlcNAc oxazoline for efficient transglycosylation of deglycosylated IgG.[42] To address this apparent discrepancy of observations, we re-evaluated the transglycosylation efficiency of wild-type EndoS at a lower temperature (4 °C) using a much less quantity of enzyme, following the recent report.[42] Using this modified condition, we did observe significant transglycosylation of the deglycosylated rituximab (**1**) with the complex sugar oxazoline (**2**) by the wild-type EndoS at the initial incubation period, but the product was gradually hydrolyzed when the incubation continued (Figure S5, Supporting Information). Thus, the reaction condition should be carefully controlled in order to trap the transglycosylation product when wild-type EndoS is used. For practical application, the EndoS glycosynthase mutants should be the choice for efficient and complete transglycosylation, as they are devoid of product hydrolytic activity.

Glycoengineering of Rituximab To Provide Nonfucosylated and Galactosylated G2 Glycoform. For anticancer therapy, nonfucosylated IgG glycoforms are desirable as it has been previously demonstrated that mAbs with low-fucose contents of Fc *N*-glycans showed enhanced ADCC activity in vitro and enhanced anticancer efficacy in vivo, particularly for those patients carrying the low affinity F158 allele of the FcγIIIa receptor.[16−19,52] No efficient method was available to efficiently transform an existing fucosylated mAb (the major glycoform of recombinant mAbs produced in mammalian cells) to a nonfucosylated mAb. To address this issue, we tested a series of commercially available α-fucosidases, but none could remove the α1,6-fucose in the intact rituximab (Scheme 2). These results implicate that the α-1,6-fucose moiety might be shielded by the Fc domain and/or the complex *N*-glycan, making it inaccessible to α-fucosidases. We reasoned that, upon deglycosylation, the resulting Fuc(α1,6)-GlcNAc glycoform of rituximab might be more accessible to α-fucosidases. Accordingly, we tested the activity of several commercially available α-fucosidases on the deglycosylated rituximab (**1**) that carries only the Fuc(α1,6)GlcNAc moiety. It was found that a nonspecific α-fucosidase from bovine kidney did have a moderate activity and was able to remove the fucose residue from the deglycosylated rituximab (**1**) to give the GlcNAc-containing rituximab (**8**) (Figure S6, Supporting Information). Although a relatively large amount of α-fucosidase and a prolonged reaction time were needed to achieve a complete defucosylation of the EndoS-deglycosylated rituximab due to the moderate activity of the α-fucosidase, the discovery of this α-fucosidase activity provides an alternative way to obtain the defucosylated rituximab precursor (**8**) for further glycoengineering.

Next, we found that the glycosynthases EndoS-D233A and EndoS-D233Q were also efficient to recognize the nonfucosylated GlcNAc in **8** for transglycosylation with an asialylated *N*-glycan oxazoline (**9**)[38] to provide the homogeneous, nonfucosylated G2 glycoform (**10**) in an essentially quantitative conversion (Scheme 2). The product was purified by protein A affinity chromatography. The identity and purity of the glycoengineered product (**10**) were confirmed by SDS-PAGE and LC-MS analysis (Figure 4). The defucosylated rituximab (**8**) showed a single species at 49 274 (Figure 4b), confirming the removal of the fucose (calcd. for the heavy chain of GlcNAc−rituximab, M = 49 274 Da). The deconvoluted ESI-MS of the heavy chain of the transglycosylation product (**10**) appeared as a single species at 50 695 (Figure 4c), which matched well with the calculated molecular mass (M = 50 693 Da) of the rituximab heavy chain carrying an asialylated biantennary complex type *N*-glycan, Gal2GlcNAc2Man3GlcNAc2. In a comparative study, we also found that, while

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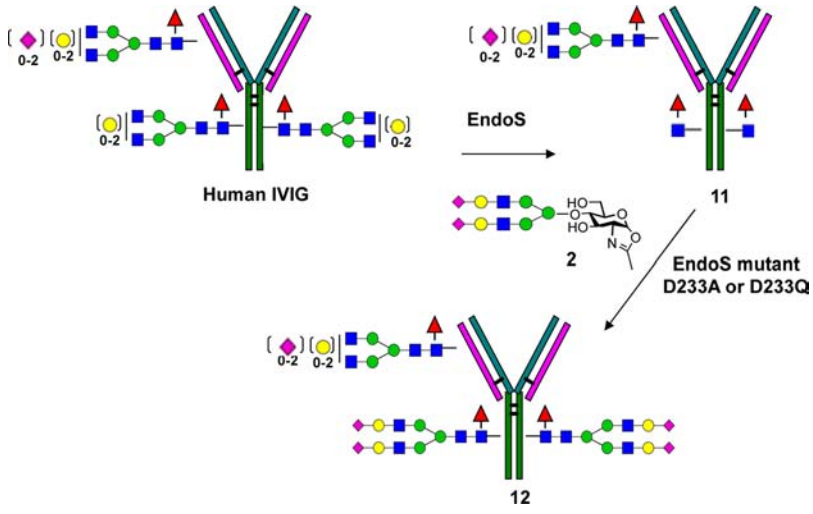
Scheme 3. Site-Specific Fc Glycoengineering of Human IVIG



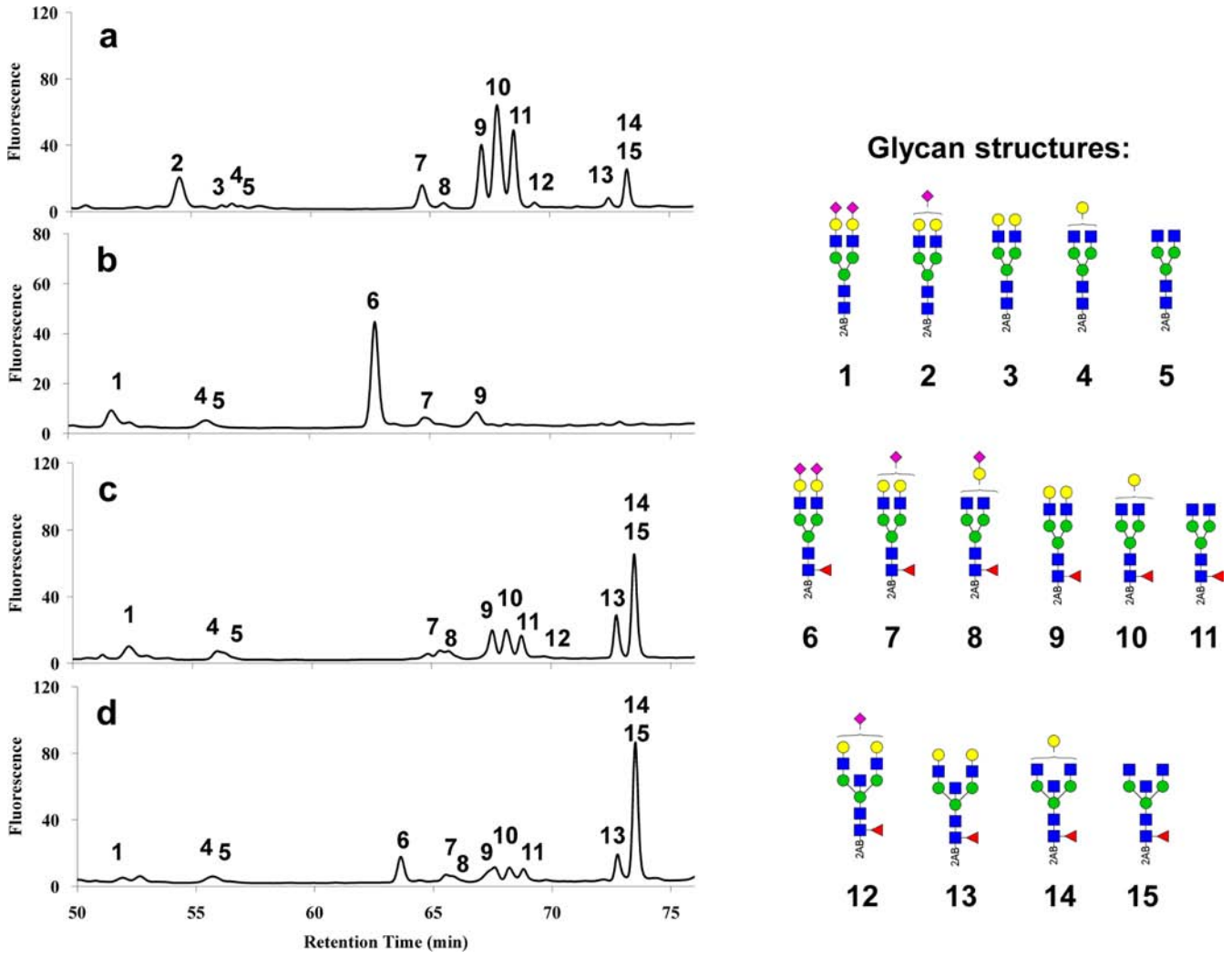


Figure 5. Fluorescent HPLC profiles of 2-AB-labeled *N*-glycans from Fab and Fc of IVIG (a) from native IVIG Fc, (b) from glycoengineered IVIG Fc, (c) from native IVIG Fab, and (d) from glycoengineered IVIG Fab.

mutants D233A and D233Q recognized both the fucosylated GlcNAc–rituximab (**1**) and the nonfucosylated GlcNAc–rituximab (**8**) as acceptors for transglycosylation, the two glycosynthase mutants preferred the fucosylated GlcNAc–rituximab (**1**) as acceptor, with a faster transglycosylation reaction than the nonfucosylated acceptor (**8**) (data not shown). Taken together, these experimental results revealed a combined enzymatic approach to making the nonfucosylated

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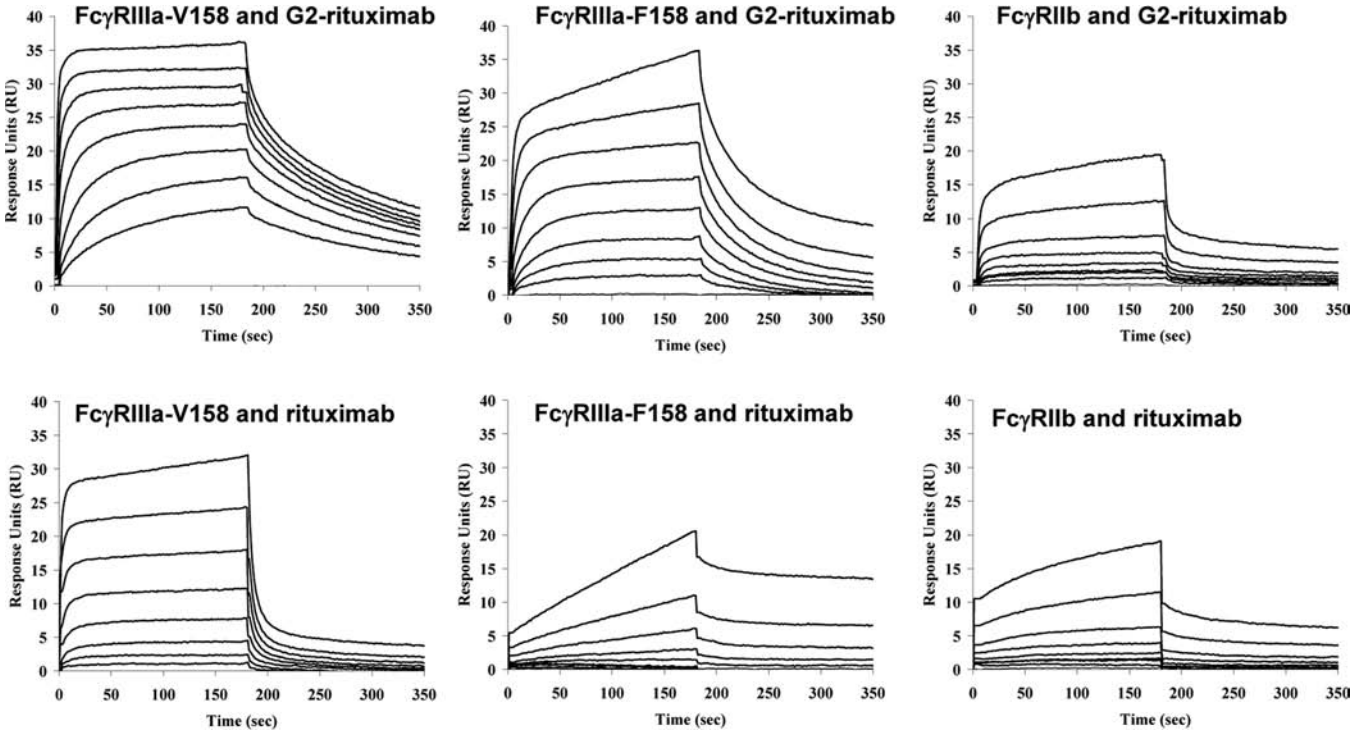

Figure 6. Typical SPR sensorgrams of the binding of G2−rituximab and commercial rituximab with respective Fcγ receptors: FcγRIIIa-V158, FcγRIIIa-F158, and FcγRIIb. The antibodies were immobilized by protein A capture, and the binding was analyzed by injecting the respective Fcγ receptors at serial two-fold dilutions starting at 40 μg/mL (1.33 μM).

and fully galactosylated homogeneous glycoform from commercially available monoclonal antibodies. The resulting nonfucosylated and galactosylated rituximab is expected to gain improved ADCC and CDC effector functions, as suggested by previously studies.[2,16−20,52]

Site-Selective Fc Glycoengineering of IVIG To Provide Fully Fc Sialylated IVIG Glycoforms. The successful glycosylation remodeling of rituximab prompted us to examine this chemoenzymatic method for glycoengineering of IVIG aiming to enhance its anti-inflammatory activity. IVIG is a pooled IgG fractions purified from the plasma of thousands of healthy donors. Recent studies have suggested that a minor, α2,6-sialylated Fc glycoform is the active species in IVIG that confers anti-inflammatory activity as demonstrated in a mouse model of rheumatoid arthritis.[21,22,53,54] Since the sialylated Fc glycoforms are minor components in IVIG,[55] the dependence of IVIG's anti-inflammatory activity on terminal Fc sialylation may partially explain why a high dose (1−2 g/kg) of infusion of IVIG is required for conferring protection. Direct sialylation of Fc and IVIG was attempted using human α-1,6-sialyltransferase (ST6Gal-I) but the efficiency was low, and in most cases, only monosialylated glycoforms were obtained as the major products.[22,56] Moreover, approximately 30% of the FAB domains in IVIG are *N*-glycosylated and lectin enrichment of Fc sialylated glycoforms of IVIG would be less efficient when the FAB glycans are sialylated.[2,57] Therefore, it would be highly desirable if Fc-specific glycoengineering with sialylated *N*-glycans can be achieved without altering the FAB glycosylation.

We found that EndoS was able to selectively deglycosylate the Fc domain of IVIG without hydrolyzing the *N*-glycans at the FAB domains under a mild condition. Moreover, the deglycosylated Fc domain of IVIG (**11**) could be selectively glycosylated with a sialoglycan oxazoline (**2**) by the EndoS-D233Q mutant to give the Fc fully sialylated IVIG (**12**) (Scheme 3). The glycoengineering was first monitored by SDS-PAGE analysis. The deglycosylation and reglycosylation of IVIG were apparent as shown in the change of the band size of the heavy chain (Figure S7, Supporting Information). To further characterize the site-selectivity of the glycoengineering of IVIG, the FAB and Fc domains were disconnected by papain digestion.[58] The Fc domain was isolated by protein A affinity chromatography and the FAB domains left in the flow-through were isolated by size exclusion chromatography on a fast protein liquid chromatography (FPLC) system. Then, the Fc and FAB *N*-glycans were released separately by PNGase F treatment, labeled with 2-aminobenzamide (2-AB),[59] and analyzed by HPLC (fluorescent detection and quantitation) and MS characterization. The FAB and Fc *N*-glycan profiles before and after glycoengineering of IVIG were shown in Figure 5. It was found that the Fc glycosylation patterns of IVIG were more complex than the Fc glycosylation of monoclonal antibody rituximab. In addition to G0F, G1F, and G2F glycoforms as the major components, there were a significant amount of monosialylated (peaks 2 and 7) glycoforms (approximately 10%) and bisecting GlcNAc-containing glycoforms (peaks 13−15) (5%) (Figure 5a). The Fc glycosylation after glycoengineering (through EndoS-deglycosylation and subsequent transglycosylation with sialoglycan oxazoline **2** by EndoS-D233Q) showed the fully sialylated glycans (peaks 1 and 6) as the major glycoforms (>90%) (Figure 5b). Interestingly, the FAB glycosylation patterns were similar before and after the glycoengineering process (compare Figure 5 c and d), except the generation of a small amount of the fully sialylated glycoform (peak 6). These results indicate that the EndoS-based glycosylation remodeling process is highly selective for the Fc *N*-glycans of intact IgG

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antibodies even in the presence of FAB glycosylation. The remarkable selectivity and high efficiency of the present Fc glycoengineering approach provide a novel avenue to transforming the commercial IVIG into fully Fc-sialylated IVIG preparation that is expected to exhibit enhanced anti-inflammatory activity, as demonstrated in previous studies using a mouse model.[21,22,53,54]

Binding of the Glycoengineered Rituximab to the Stimulatory Fcγ Receptor (FcγRIIIa) and the Inhibitory Fcγ Receptor (FcγRIIb). The affinity of the remodeled glycoforms of rituximab for respective Fcγ receptors (FcγRIIIa-F158, FcγRIIIa-V158, and FcγRIIb) was examined by surface plasmon resonance (SPR) analysis. The rituximab glycoforms were site-specifically immobilized on a protein A chips and the Fcγ receptors at various concentrations were injected as analytes, following our recently reported procedures.[35] As expected, the nonfucosylated G2 glycoform showed significantly enhanced affinity for both the low-affinity and high-affinity FcγIIIa receptors, FcγRIIIa-F158 and FcγRIIIa-V158, when compared with the commercially available rituximab (Figure 6). The K_D values for the binding of the G2 glycoform (**10**) to the FcγRIIIa-F158 and FcγRIIIa-V158 were 123 ± 11 and 12 ± 2 nM, respectively, which were obtained by fitting the binding data with a 1:1 steady-state model using the BIAcore T100 evaluation software. On the other hand, the K_D values for the binding of the commercial rituximab to the FcγRIIIa-F158 and FcγRIIIa-V158 were estimated to be 1042 ± 155 and 252 ± 18 nM, respectively. Thus, the affinity of the glycoengineered G2 glycoform for the low-affinity and high-affinity Fcγ receptors (FcγRIIIa-F158 and FcγRIIIa-V158) was about 9-fold and 20-fold higher than the commercial rituximab, respectively. On the other hand, the G2 glycoform and the commercial rituximab demonstrated comparable affinity for the inhibitory Fcγ receptor FcγRIIb with the K_D values of 2.3 ± 0.5 and 2.0 ± 0.7 μM, respectively. These results reveal a clear gain of beneficial functions for the glycoengineered rituximab. It should be pointed out that an efficient preparation of high-affinity FcγRIIIa-binding glycoforms is clinically significant to address the issue of Fcγ receptor polymorphism found in cancer patients who are less or not responsive to the treatment with common MAbs. In these patients, their FcγRIIIa-F158 allele has a low affinity to the therapeutic mAbs such as rituximab in comparison with the high-affinity receptor, FcγRIIIa-V158 allele.[52,60,61] Fcγ receptor-mediated effector functions were also suggested to be an important mechanism for achieving protective immunity for HIV-neutralizing antibodies.[62] Thus, the glycoengineering approach described here may find wide applications in producing various defined glycoforms of monoclonal antibodies valuable for functional studies as well as for biomedical applications.

CONCLUSION

An efficient chemoenzymatic approach to glycoengineering of intact IgG antibodies is described. The two new EndoS-based glycosynthases generated by site-directed mutagenesis demonstrate broad substrate specificity capable of transferring sialylated and asialylated and complex type *N*-glycans as well as selectively modified *N*-glycan core from the corresponding glycan oxazolines to Fc-deglycosylated intact antibodies. In addition, the deglycosylation/reglycosylation approach is efficient for both core-fucosylated and nonfucosylated IgG antibodies when an α-fucosidase is adequately combined. These new findings significantly expand the scope of the chemoenzymatic method and made possible an efficient transformation of intact monoclonal antibodies into various well-defined glycoforms that are hitherto difficult to obtain by existing methods. It is expected that this glycoengineering approach may facilitate the development of biosimilar and/or biobetter biologics that possess improved therapeutic efficacy and/or gain new functions.

EXPERIMENTAL SECTION

Materials. Monoclonal antibody rituximab (rituxan, Genentech Inc., South San Francisco, CA) and IVIG were purchased through Premium Health Services Inc. (Columbia, MD). Sialoglycan oxazoline (**2**) and asialo-complex-type glycan oxazoline (**5**) were synthesized following our previously reported procedure.[38,46] Bovine kidney α-L-fucosidase was purchased from Sigma (St. Louis, MO) and Prozyme (Hayward, CA). Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (EndoA) and endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (EndoM) and their mutants were overproduced in *E. coli* following the reported procedures.[38] PNGase F was purchased from New England Biolabs (Ipswich, MA).

Liquid Chromatography Mass Spectrometry (LC-MS). The LC-MS was performed on a LXQ system (Thermo Scientific) with a Hypersil GOLD column (1.9 μm, 50 × 2.1 mm). The IgG samples were treated with 0.5% β-mercaptoethanol and heated at 60 °C for 15 min then subject to LC-MS measurement. The analysis was performed at 60 °C eluting with a linear gradient of 10−40% MeCN containing 0.1% formic acid within 10 min at a flow rate of 0.25 mL/min.

Electron Spray Ionization Mass Spectrometry (ESI-MS) and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). The ESI-MS spectra were measured on a Waters Micromass ZQ-4000 single quadruple mass spectrometer. The MALDI-TOF MS was performed on an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). The instrument was calibrated by using ProteoMass Peptide MALDI-MS calibration kit (MSCAL2, Sigma/Aldirich). The matrix of 2,5-dihydroxybenzoic acid (DHB) was used for the neutral glycans and 2′,4′,6′-trihydroxyacetophenone (THAP) was used for the acidic glycans.

Overexpression and Purification of EndoS and Mutants. Wild-type EndoS was overproduced in *E. coli* and purified according to the previously reported procedures,[40,63] using the plasmid pGEX-EndoS that was kindly provided by Dr. M. Collin (Lund University, Sweden). The two EndoS mutants, D233A and D233Q, were generated using the GENEART site-directed mutagenesis kit (Invitrogen) per the manufacturer's directions. The pGEX-EndoS plasmid was used as the template, and LA Taq polymerase (Takara, Japan) was used for PCR. Mutations were confirmed by DNA sequencing and transformed into BL21(DE3). The transformants were cultured in Luria−Bertani medium containing 100 mg/L carbenicillin and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 16 h at 25 °C. The cells were harvested by centrifugation at 1700*g* for 15 min at 4 °C. The cell pellet was suspended in phosphate-buffered saline (pH 7.4) with lysozyme and PMSF. The lysed mixture was centrifuged at 16 000*g* for 20 min at 4 °C. After centrifugation, the supernatant from the cell lysis was applied to 3 mL of 50% glutathione−Sepharose 4B resin (GE Healthcare). Samples were incubated at 25 °C for 60 min with gentle rocking. The resin was applied to a 10 mL column (PD-10 GE Healthcare) and washed five times with PBS. 500 μL of glutathione elution buffer (50 mM Tris−HCl, 10 mM glutathione, pH 8.0) was added to the column, incubated at room temperature for 5 min, collected, and then repeated three times. The eluted fractions were pooled and dialyzed against sodium phosphate buffer (50 mM, pH 7.0) overnight at 4 °C. Protein samples were then concentrated using Amicon ultra centrifugal filters 10 kDa (Millipore). Concentrated protein samples were analyzed by SDS-PAGE, and protein concentration was quantified using a Nano-Drop 2000c spectrophotometer. The yield of overproduction of the wild-

type EndoS was approximately 40 mg/L, and the yield for the mutants was approximately 30 mg/L.

Deglycosylation of Rituximab by Wild-Type EndoS To Give (Fucα1,6)GlcNAc−Rituximab (1). Commercial rituximab (20 mg) in a Tris−Cl buffer (50 mM, pH 8.0, 2 mL) was incubated with EndoS (30 μg) at 37 °C for 1 h. LC-MS and SDS-PAGE analyses indicated the complete cleavage of the *N*-glycans on the heavy chain. The reaction mixture was subject to affinity chromatography on a column of protein A−agarose resin (5 mL) that was pre-equilibrated with a Tris−Cl buffer (20 mM, pH 8.0). The column was washed with Tris−Cl (20 mM, pH 8.0, 25 mL) and glycine−HCl (20 mM, pH 5.0, 20 mL) successively. The bound IgG was released with glycine−HCl (100 mM, pH 2.5, 20 mL), and the elution fractions were immediately neutralized with Tris−Cl buffer (1.0 M, pH 8.8). The fractions containing the Fc fragments were combined and concentrated by centrifugal filtration (Amicon Ultra centrifugal filter, Millipore, Billerica, MA) to give (Fucα1,6)GlcNAc−rituximab (**1**) (18 mg). LC-MS: calculated for the heavy chain of (Fucα1,6)GlcNAc-rituximab (**1**), *M* = 49 420 Da;[47] found (*m*/*z*), 49 420 (deconvolution data).

Transglycosylation of (Fucα1,6)GlcNAc−Rituximab (1) with Sialoglycan Oxazoline (2) by EndoS Mutants D233A or D233Q. A solution of (Fucα1,6)GlcNAc−rituximab (**1**) (10 mg) and sialoglycan−oxazoline (**2**) (10 mg) in a Tris buffer (50 mM, pH 7.4, 2 mL) was incubated with the EndoS mutant D233A or D233Q (200 μg) at 30 °C. Aliquots were taken at intervals and were analysis by LC-MS. After 2−3 h, LC-MS monitoring indicated the complete reaction of (Fucα1,6)GlcNAc−rituximab (**1**) to give the transglycosylation product (**3**) carrying the fully sialylated *N*-glycans. The reaction mixture was subject to an affinity chromatography on a protein A−agarose column following the procedure described above. Fractions containing the product were combined and concentrated by ultracentrifugation to give sialylated rituximab (**3**) (11 mg, quantitative). LC-MS: calculated for the heavy chain of **3** carrying the fully sialylated *N*-glycan, *M* = 51 421 Da; found (*m*/*z*), 51 426 (deconvolution data).

Transglycosylation of (Fucα1,6)GlcNAc−Rituximab (1) with Man3GlcNAc Oxazoline (4) and the Azide-Tagged Man3GlcNAc Oxazoline (6) by EndoS-D233Q. The transglycosylation was performed as described for the preparation of **3** to give the corresponding products. LC-MS analysis of glycoengineered rituximab **5** and **7**: calculated for the heavy chain of **5** carrying the fucosylated Man3GlcNAc2 *N*-glycan, *M* = 50 109 Da; found (*m*/*z*), 50 112 (deconvolution data); calculated for the heavy chain of **7** carrying the fucosylated azido-Man3GlcNAc2 *N*-glycan, *M* = 50 134 Da; found (*m*/*z*), 50 143 (deconvolution data).

Defucosylation of (Fucα1,6)GlcNAc−Rituximab (1) by Bovine Kidney α-Fucosidase. A solution of (Fucα1,6)GlcNAc−rituximab (**1**) (2 mg) in a phosphate buffer (50 mM, pH 5.5, 200 μL) containing 0.05 sodium azide was incubated with the fucosidase from bovine kidney (Prozyme, 5 U) at 37 °C. Aliquots were taken at intervals and were analyzed by LC-MS. After 20 days, LC-MS monitoring indicated the complete defucosylation of (Fucα1,6)-GlcNAc-rituximab (**1**) to give the product, GlcNAc−rituximab (**2**). The reaction mixture was subject to affinity chromatography on a column of protein A following the procedure described above. Fractions containing the product were combined and concentrated by ultracentrifugation to give GlcNAc−rituximab (**2**) (2 mg, quantitative). LC-MS: calculated for the heavy chain of GlcNAc−rituximab (**2**) carrying a GlcNAc moiety, *M* = 49 274 Da; found (*m*/*z*), 49 274 (deconvolution data).

Transglycosylation of GlcNAc−Rituximab (4) with Asialylated Complex-Type Glycan Oxazoline (5) by D233Q Mutant. A solution of GlcNAc−rituximab (**4**) (2 mg) and oxazoline **5** (5 mg) in a Tris buffer (50 mM, pH 7.4, 0.5 mL) was incubated with the EndoS-D233Q (200 μg) at 37 °C. Aliquots were taken at intervals and were analyzed by LC-MS. After 2 h, LC-MS monitoring indicated the complete reaction of **4** to give the corresponding transglycosylation product (**6**). The reaction mixture was subject to affinity chromatography on a column of protein A. Fractions containing the product were combined and concentrated by ultracentrifugation to give the nonfucosylated rituximab glycoform (**6**) (2 mg, quantitative). LC-MS: calculated for the heavy chain of **6** carrying the nonfucosylated *N*-glycan, *M* = 50 693 Da; found (*m*/*z*), 50 695 (deconvolution data).

Site-Specific Deglycosylation at the Fc Domain of IVIG by EndoS. Commercial IVIG (20 mg) in a Tris−Cl buffer (50 mM, pH 8.0, 2 mL) was incubated with EndoS (30 μg) at 37 °C for 1 h. The residue was subject to affinity chromatography on a column of protein A to give the (Fucα1,6)GlcNAc−IVIG (20 mg, quantitative), in which the Fc *N*-glycans were removed leaving the α1,6-fucosylated GlcNAc at the N297 sites.

Transglycosylation of (Fucα1,6)GlcNAc−IVIG with Sialoglycan Oxazoline (2) by D233Q Mutant. A solution of (Fucα1,6)-GlcNAc−IVIG (3 mg) and sialoglycan−oxazoline (**2**) (3 mg) in a Tris buffer (50 mM, pH 7.4, 2 mL) was incubated with the D233Q mutant (60 μg) at 30 °C. After 2 h, SDS-PAGE analysis indicated the complete reaction of (Fucα1,6)GlcNAc−IVIG to give the transglycosylation product. The reaction mixture was subject to affinity chromatography on a column of protein A to provide the glycoremodeled IVIG (3 mg, quantitative), in which the Fc *N*-glycans were remodeled to the fully sialylated complex type *N*-glycans.

Surface Plasmon Resonance (SPR) Binding Experiments. The binding between different glycoforms of IgG and Fcγ receptors was measured by surface plasmon resonance (SPR) using a Biacore T100 instrument (GE Healthcare, USA). Protein A of 5000 RU was immobilized on a CM5 biosensor chip (GE Healthcare) using a standard primary amine coupling chemistry at pH 4.5 to capture the different glycoforms of IgG. A reference flow cell was prepared similarly without injecting protein A. Each individual glycoform of IgG in HBS-P buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 0.05% v/v surfactant P20) was injected at 10 μL/min onto the protein A surface and reached the capture level of 150 RU. A serial dilution of FcγIIIa and FcγIIb receptors was injected at 10 μL/min. After each cycle, the surface was regenerated by injecting 10 mM HCl at 10 μL/min for 30 s. Data were fitted into a 1:1 Langmuir binding model using BIAcore T100 evaluation software to obtain the equilibrium constant (K_D) data.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Complete ref 29; MALDI-TOF MS, LC-MS, and SDS-PAGE analysis of *N*-glycans, monoclonal antibody rituximab, and IVIG during the glycoengineering process. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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