Endo-F3 Glycosynthase Mutants Enable Chemoenzymatic Synthesis of Core-fucosylated Triantennary Complex Type Glycopeptides and Glycoproteins*

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Chemoenzymatic synthesis is emerging as a promising approach to the synthesis of homogeneous glycopeptides and glycoproteins highly demand for functional glycomics studies, but its generality relies on the availability of a range of enzymes with high catalytic efficiency and well defined substrate specificity. We describe in this paper the discovery of glycosynthase mutants derived from Elizabethkingia meningoseptica endoglycosidase F3 (Endo-F3) of the GH18 family, which are devoid of the inherent hydrolytic activity but are able to take glycans oxazolines for transglycosylation. Notably, the Endo-F3 D165A and D165Q mutants demonstrated high acceptor substrate specificity toward α1,6-fucosyl-GlcNAc-Asn or α1,6-fucosyl-GlcNAc-polypeptide in transglycosylation, enabling a highly convergent synthesis of core-fucosylated, complex CD52 glycopeptide antigen. The Endo-F3 mutants were able to use both bi- and triantennary glycan oxazolines as substrates for transglycosylation, in contrast to previously reported endoglycosidases derived from Endo-S, Endo-M, Endo-D, and Endo-A mutants that could not recognize triantennary N-glycans. Using rituximab as a model system, we have further demonstrated that the Endo-F3 mutants are highly efficient for glycosylation remodeling of monoclonal antibodies to produce homogeneous intact antibody glycoforms. Interestingly, the new triantennary glycan glycoform of antibody showed much higher affinity for galexin-3 than that of the commercial antibody. The Endo-F3 mutants represent the first endoglycosidase-based glycosynthases capable of transferring triantennary complex N-glycans, which would be very useful for glycoprotein synthesis and glycosylation remodeling of antibodies.

Protein glycosylation is one of the most prevalent posttranslational modifications, found in almost all living organisms ranging from bacteria to eukaryotes (1). Glycosylation can profoundly affect a protein’s intrinsic properties, such as folding, stability, intracellular trafficking, and immunogenicity. In addition, the oligosaccharide components of glycoproteins can participate directly in a number of important biological recognition processes, including cell adhesion, signaling, host-pathogen interactions, and immune responses (2–8). It is well documented that subtle changes in glycosylation can lead to a significant impact on the biological functions and, in the case of therapeutic glycoproteins, such as monoclonal antibodies, the in vivo stability and therapeutic efficacy (7, 9–12). Natural and recombinant glycoproteins are usually produced as mixtures of glycoforms that differ only in the structures of pendant glycans, from which pure glycoforms are extremely difficult to isolate by current chromatographic techniques. As a result, synthetic homogeneous glycopeptides and glycoproteins emerge as indispensable tools for functional studies and for drug/vaccine discoveries. Many elegant chemical and biochemical strategies have been explored for making homogeneous glycoproteins and mimics, including total chemical synthesis with native chemical ligation (13–17), chemoselective ligation (18, 19), chemoenzymatic synthesis (20–24), and glycosylation pathway engineering in host expression systems (25–28). As part of these efforts, we have attempted to develop a chemoenzymatic method for construction of complex N-glycopeptides and glycoproteins that is based on the transglycosylation activity of a class of endo-β-N-acetylglucosaminidases (the endoglycosidases that hydrolyze N-glycans of glycoproteins) for convergent native ligation of preassembled glycans and GlcNAc-peptide/protein (20, 23, 24). The success of this approach relies on the availability of novel endoglycosidase-derived glycosynthase mutants that are devoid of hydrolysis activity but can use the highly activated glycan oxazolines as substrates for transglycosylation. Several endoglycosidases have been successfully converted into glycosynthases via site-directed mutation of a critical residue that appears to be essential in promoting the formation of the sugar oxazolinium ion intermediate at the catalytic site during enzymatic hydrolysis. These include Endo-A³ (from bacterium Arthrobacter protophormiae), Endo-M (from fungus Mucor hiemalis), and Endo-D (from bacterium Strepto-

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3 The abbreviations used are: Endo, endo-β-N-acetylglucosaminidase; CPD, cysteine protease domain; CT, complex type; ESI, electron spray ionization; Fuc, fucose; GlcNAc, N-acetylglucosamine; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; HSQC, heteronuclear single quantum coherence spectroscopy; PNGase F, peptide-N-glycosidase F; SCT, sialylated complex type; SPR, surface plasmon resonance; TCT, triantennary complex type; ox, oxazoline; GH, glycoside hydrodolase; Fmoc, N-(9-fluorenylmethoxycarbonyl); SGP, sialylglycopeptide.
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coccus pneumoniae) of the glycoside hydrolase (GH) family 85 as well as Endo-S (from Streptococcus pyogenes) of the GH family 18 (29–34). These enzymes and related mutants showed remarkably different substrate specificity in transglycosylation. For example, the glycosynthase mutants derived from Endo-M (such as Endo-M N175Q) are efficient for transferring biantennary complex type and high mannose type N-glycans to GlcNAc-peptide/protein acceptors but are unable to accept α1,6-fucose-substituted (core-fucosylated) GlcNAc-peptide/protein as acceptor substrates; nor can they efficiently glycosylate Fc domains or intact antibodies (29–31, 35–38). The glycosynthases derived from Endo-S (such as the Endo-S D233A and D233Q mutants) appear to be highly specific for glycosylation of the Fc domain of antibodies (33, 39, 40), but they show only marginal activity for regular GlcNAc-peptide/protein acceptors. So far, no glycosynthases in this category have been reported that are able to transfer highly branched N-glycans, such as triantennary N-glycans, for glycoprotein synthesis. In addition, although Endo-D mutants (such as Endo-D D322A) have been shown to accept Fucα1,6GlcNAc-peptide as acceptor for transglycosylation, they are limited to transferring only the N-glycan core and are unable to accommodate common complex type N-glycans for transglycosylation (34).

Core fucosylation, the attachment of a fucose moiety to the innermost GlcNAc moiety of the N-linked glycans in an α1,6-glycosidic linkage, is a modification frequently found in natural and recombinant glycoproteins. It has been reported that core fucosylation affects the conformations of N-glycans and modulates glycan-lectin interactions and antibody-Fc receptor recognition (41–45). Moreover, core fucosylation is associated with cancer progression, suggesting that core-fucosylated N-glycans and glycopeptides could serve as novel biomarkers for cancer diagnosis (46, 47). Despite remarkable advances in synthetic methodology, synthesis of sialylated and fucosylated glycans, and recombinant glycoproteins. It has been reported that core fucosylation product. In this paper, we report the generation of glycosynthase mutants from Endo-F3 by site-directed mutagenesis of the putative catalytic residue, Asp-165, which is assumed to play a role in promoting the formation of sugar oxazolion ion intermediate in hydrolysis. Our experimental data reveal that the Endo-F3 mutants, including D165A and D165Q, are typical glycosynthases that are able to efficiently transfer both bi- and triantennary complex type N-glycans (in the form of glycan oxazolines) to fucosylated GlcNAc-peptide acceptors to form core-fucosylated complex glycopeptides with high efficiency. The Endo-F3 mutants D165A and D165Q represent the first endoglycosidase-based glycosynthases capable of transferring triantennary complex type N-glycans for glycopeptide synthesis and glycosylation remodeling of core-fucosylated glycoproteins, including therapeutic antibodies.

Experimental Procedures

Materials—Fmoc-Asn(Fucα1,6GlcNAc)-OH and core-fucosylated CD52 antigen were synthesized following our previously reported procedures (56). Monoclonal antibody rituximab (rituxan, Genetech Inc., South San Francisco, CA) was purchased through Premium Health Services Inc. (Columbia, MD). Asialo and sialoglycan complex type oxazoline was synthesized following a modified reported procedure (57). Endo-S WT and Endo-S D233A from S. pneumoniae were overexpressed and purified following our previous procedure (33). Fetuin was purified from the fetal bovine serum (Sigma) using a modified procedure of Spiro (58). Porcine fibrinogen was purified from porcine plasma (Sigma) using a modified procedure (59).

Cloning, Expression, and Purification of Endo-F3—The cDNA fragment encoding the Endo-F3 gene without the signal peptide (nucleotides 118–987; amino acids 40–329) was amplified by PCR from the genomic DNA of E. meningoseptica (ATCC number 51720D). The forward primer was 5’-GGAATTCCTATGGCTACTGCTTTGGCGG, and the reverse primer was 5’-CCCGGATCCAGGTTCTTCACTG- CATCCTC. An Ndel site was added to the forward primer and a BamHI site was added to the reverse primer (underlined). Both amplified DNA fragments were cloned into pCPD-Lasso (a PET22b-CPD derivative) after digestion with Ndel and BamHI. The constructed plasmid, pCPD-Lasso Endo-F3, was transformed into BL21 (DE3). The transformants were cultured in 1 liter of terrific broth medium supplemented with 50 μg/ml carbenicillin. Cultures were grown at 37 °C until the cells reached an A_600 nm of 1–1.5. Then 0.1 mM isopropyl β-D-1-thiogalactopyranoside and 100 ml of glycerol were added to the culture to induce protein overproduction at 16 °C. After 30 h, the cells were harvested by centrifugation. The cell pellets were lysed using B-PER protein extraction reagent (Pierce) following the manufacturer’s instructions. The cell lysis was applied to HisTrap HP (GE Healthcare) and washed with phosphate-buffered saline (PBS) with 30 mM imidazole (pH 7.4). Bound His-tagged protein was eluted with a gradient from 0 to 500 mM imidazole in PBS buffer. The purified Endo-F3 was desalted and concentrated with PBS using centrifugal diafiltration with an Amicon Ultra filtration unit (10 kDa; Millipore). The protein purity was confirmed using SDS-PAGE, and the concentration was quan-
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Using measurement of the absorption at 280 nm on a NanoDrop 2000c.

High Performance Liquid Chromatography (HPLC)—Analytical RP-chromatography was performed on a Waters 626 HPLC instrument and a Symmetry 300 C18 column (5 μm, 4.6 × 250 mm) or an XBridge BEH130 C18 column (3.5 μm, 4.6 × 250 mm) at 40°C. The Symmetry300 column was eluted with a linear gradient of acetonitrile (0–60%) containing TFA (0.1%) over 30 min at a flow rate of 1.0 ml/min. (method A). The XBridge column was eluted with a linear gradient of acetonitrile (0–20%) containing TFA (0.1%) over 30 min at a flow rate of 0.5 ml/min (method B).

Liquid Chromatography Mass Spectrometry (LC-ESI-MS) of Free Glycan and Glycopeptides—The LC-MS was performed on a LXQ Linear Ion Trap or Exactive Plus Orbitrap (Thermo Scientific) with an Agilent Poroshell 300SB-C8 column (5 μm, 75 × 1 mm). The analysis was performed at 30°C, eluting with a linear gradient of 0–10% acetonitrile containing 0.1% formic acid within 10 min at a flow rate of 0.40 ml/min.

LC-ESI-MS of IgG—The LC-MS analysis was performed on a LXQ Linear Ion Trap or Exactive Plus Orbitrap (Thermo Scientific) with an Agilent Poroshell 300SB-C8 column (5 μm, 75 × 1 mm). The IgG antibody samples were treated with 50 mM tris(2-carboxyethyl)phosphine and heated at 37°C for 20 min and then subjected to LC-MS analysis. The analysis was performed at 60°C, eluting with a linear gradient of 25–35% acetonitrile containing 0.1% formic acid within 10 min at a flow rate of 0.40 ml/min. The LC-MS analysis of PNGase F-treated antibody glycoforms was performed in the same manner but included a 1-h incubation with PNGase F prior to tris(2-carboxyethyl)phosphine treatment. Raw data were deconvoluted using MagTran (Amgen). The molecular mass of the commercial rituximab was calculated using the amino acid sequence described in drugbank.ca with the following known modifications: N-terminal pyroglutamic acid formation on the light and heavy chain, C-terminal lysine cleavage of the heavy chain, and full reduction of intrachain disulfide bonds (two in the light chain and four in the heavy chain).

High Performance Anion Exchange Chromatography Coupled with Pulsed Amperometric Detection (HPAEC-PAD)—HPAEC-PAD was performed on a Dionex DX600 or Dionex ICS-5000 chromatography system (Fisher) equipped with an electrochemical detector (EDso) and an anion exchange column (CarboPac PA100 4 × 250 mm or CarboPac PA200, 4 × 250 mm). The mobile phase (flow rate, 0.5 ml/min) was composed of 100 mM NaOH (elucent A) and 250 mM NaOAc, 100 mM NaOH (elucent B). The gradient used was as follows: 0 min, 49.5% eluent A, 0.5% eluent B, 50% eluent C; 10.0 min, 45% eluent A, 5% eluent B, 50% eluent C; and 50.0 min, 27.5% eluent A, 22.5% eluent B, and 50% eluent C.

NMR—The purified N-glycans were deuterium-exchanged using 99% D2O (three times) and dried by lyophilization. The dried glycans were dissolved in 500 μl of deuterium oxide (100%) and transferred to an NMR tube. Two-dimensional HSQC 1H/13C NMR spectra were recorded in a Bruker 950-MHz NMR system with a cryo-probe. All chemical shifts were assigned in ppm.
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1H NMR (D2O, 950 MHz) of TCT oxazoline 7: δ 6.11 (H-1 of GlcNAc 2), 5.12 (H-1 of α-Man 4), 4.96 (H-1 of α-Man 4'), 4.74 (H-1 of β-Man 3), 4.62 (H-1 GlcNAc 5), 4.58 (H-1 GlcNAc 5'), 4.56 (H-1 of GlcNAc 7), 4.48 (H-1 of Gal 6, 6°,8), 2.06 (NAC GlcNAc 2,5,7,7').

13C NMR (D2O, 239 MHz) of TCT oxazoline 7: δ 100.01 (C-1 of oxazoline), 99.12 (C-1 of α-Man4), 96.60 (C-1 of α-Man4'), 101.3 (C-1 of β-Man 3), 99.46 (C-1 GlcNAc 5'), 99.46 (C-1 GlcNAc 5, 7), 102.9 (C-1 of Gal 6, 6°,8), 22.37 (NAC GlcNAc 2,5,7,7').

Transglycosylation of Fmoc-Asn(Fuc1,6 GlcNAc)-OH with Sialylated Complex Type Glycan Oxazoline (SCTox) by Endo-F3 D165A; Synthesis of 4—A solution of SCTox (1 (125 μg, 6.24 mm), Fmoc Asn GlcNAc(Fuc)-OH (2) (10 μg, 1.422 mm), and Endo-F3 D165A (3.5 μg) was incubated at 37 °C in 100 mM Tris, pH 7.4, in a 40-μl total volume. Aliquots of 0.5 μl were taken at intervals, and the reaction was quenched with 0.1% TFA. The reaction was monitored using reverse-phase HPLC analysis (method A) along with LC-MS to confirm product formation. Calculated M = 2706.50; found m/z 903.23 [+M + 3H]- and m/z 1354.04 [M + 2H]-.

Transglycosylation of Core-fucosylated CD52 with TCTox by Endo-F3 D165A; Synthesis of 9—A solution of TCTox (6) (450 μg, 6.24 mm), CD52-GlcNAc-Fuc (100 μg, 1.56 mm) (8), and Endo-F3 D165A (14 μg) was incubated at 37 °C in 100 mM Tris, pH 7.4, in a 40-μl total volume. The reaction was monitored using reverse-phase HPLC analysis (method B). Completion conversion of 8 to 9 was observed within 2 h. The product (9) was confirmed with LC-MS. TCT-CD52 calculated M = 3384.3 Da; found (m/z), 1159.3 [M + 3H]-.

Transglycosylation of (Fuc1,6)GlcNAc-Rituximab with CTox, SCTox, and TCTox by Endo-F3 D165A; Synthesis of 12, 14, and 15—A solution of (Fuc1,6)GlcNAc-rutimubax (1 mg, 69 μm) (11) and SCTox (700 μg, 3.5 μm) (1) was incubated with Endo-F3 D165A (35 μg) at 37 °C in 100 μl of 100 mM Tris buffer (pH 7.4) for 45 min. Then another 550 μg of SCTox (1) was added, and the reaction was monitored by LC-MS of aliquots. Over incubation for a total of 1.5 h, LC-MS analysis indicated that a completion transglycosylation of the deglycosylated rutimubax was achieved. The product (12) was purified using protein A chromatography. LC-MS: calculated for the heavy chain of 12 carrying the fully sialylated biantennary N-glycan, M = 51,421 Da; found (m/z), 51,412 (deconvolution data).

A solution of (Fuc1,6)GlcNAc-rutimubax (1 mg, 69 μm) (11) and CTox (500 μg, 3.5 μm) (13) was incubated with Endo-F3 D165A (35 μg) at 37 °C in 100 μl of 100 mM Tris buffer (pH 7.4). After 45 min, another 400 μg of CTox was added. The mixture was incubated for 1 h when LC-MS analysis indicated the completion of transglycosylation. The product (15) was purified using protein A chromatography. LC-MS: for the heavy chain of carrying the complex type triantennary N-glycan (15), M = 51,204 Da; found (m/z), 51,199 (deconvolution data).

Surface Plasmon Resonance—The binding between glycoengineered IgG glycoforms and galectin-3 was measured by surface plasmon resonance (SPR) using a Biacore T100 instrument (GE Healthcare). Galectin-3 was immobilized on a CM5 biosensor chip (GE Healthcare) using amine coupling chemistry, pH 5.0, to reach 1000 response units. Each glycoform of IgG was injected at 40 μl/min. After each cycle, the surface was regenerated by injecting 3 mM MgCl2.

The binding between glycoengineered IgG glycoforms and FcyRIIa was measured by SPR using a Biacore T100 instrument (GE Healthcare). Protein A was immobilized at 6000 resonance units on a CM5 biosensor chip (GE Healthcare) using amine coupling chemistry at pH 4.5. Each glycoform of IgG in PBS was injected at 10 μl/min over the protein A surface to a capture level of 150 resonance units. A serial dilution of Fc receptor was injected at 30 μl/min. After each cycle, the surface was regenerated by injecting a glycine HCl buffer (10 mM, pH 2.5).

Results

Cloning, Expression, and Characterization of Endo-F3—Endo-F3 is a bacterial endoglycosidase from the GH18 family consisting of 329 amino acid residues (35.8 kDa) with an N-terminal signaling peptide (amino acids 1–39). Tarentino et al. (61) have previously expressed expression of Endo-F3 in Escherichia coli resulted in low yields (<1 mg/liter) of soluble enzyme due to the formation of insoluble inclusion bodies. Overexpression and refolding enabled higher yields (15 mg/liter) but required arduous solubilization with acid and detergent followed by time-consuming exhaustive dialysis (62). We initially chose to clone Endo-F3 (amino acids 40–329) into both pET41b and pET22b for expression in E. coli. Overexpression from both vectors yielded inclusion bodies of Endo-F3, but purification and refolding of the inclusion bodies following a procedure similar to one reported previously (62) gave a very low overall yield (<1 mg/liter) of soluble Endo-F3. This situation prompted us to investigate alternative methods to overexpress Endo-F3 WT in a soluble form. Previously, Shen et al. (63) have demonstrated that expression with a novel tag, the Vibrio cholerae MARTX toxin cysteine protease domain (CDP) was able to enhance the solubility and stability of recombinant proteins (63, 64). To examine whether CDP could enhance the expression of the soluble Endo-F3, we cloned the gene encoding Endo-F3 (amino acids 40–329) into a pET22b-CDP vector. This plasmid encodes the CDP domain and also includes a His10 tail. We found that the fusion protein, Endo-F3-CDP (hereafter called Endo-F3), could be efficiently expressed as a soluble protein in E. coli and was readily purified using immobilized metal ion affinity chromatography to give the soluble enzyme with a yield of >15 mg/liter.

We tested the deglycosylation activity of the recombinant Endo-F3 using several substrates, including porcine fibrinogen (a natural glycoprotein carrying core-fucosylated biantennary...
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N-glycans) (65), rituximab (a therapeutic monoclonal antibody), and the sialoglycopeptide (SGP) isolated from chicken egg yolks (Fig. 1). As revealed by SDS-PAGE and LC-MS analysis, the recombinant Endo-F3 showed potent hydrolytic activity and was able to deglycosylate porcine fibrinogen and monoclonal antibody rituximab quickly even without the need of denaturing the glycoproteins (Fig. 1, A and B). The recombinant Endo-F3 could also hydrolyze the sialoglycopeptide, SGP.

![Deglycosylation activity of recombinant Endo-F3 on natural glycoproteins and glycopeptides.](image)

**FIGURE 1.** Deglycosylation activity of recombinant Endo-F3 on natural glycoproteins and glycopeptides. A, hydrolysis of porcine fibrinogen; SDS-PAGE analysis of Endo-F3-catalyzed hydrolysis of porcine fibrinogen. Lane 1, fibrinogen; lane 2, Endo-F3 cleaved fibrinogen; lane 3, molecular weight markers. B, hydrolysis of monoclonal antibody rituximab; LC-ESI-MS analysis of the heavy chain of rituximab before and after Endo-F3 treatment. Commercial rituximab, calculated: G0F, $M = 50,514$ Da; G1F, $M = 50,676$ Da; and G2F, $M = 50,839$ Da; found, 50,507 (G0F), 50,668 (G1F), and 50,839 (G2F); rituximab after Endo-F3 treatment. Calculated heavy chain of Fuc$_{1,6}$GlcNAc-rituximab, $M = 49,418$ Da; found, 49,414 (deconvolution data). C, hydrolysis of SGP; HPAEC-PAD analysis of the EndoF3-catalyzed hydrolysis of glycopeptide SGP.
(Fig. 1C), but it was observed that the Endo-F3-catalyzed hydrolysis of SGP was much slower than that of porcine fibrinogen and rituximab, which carry core-fucosylated N-glycans. Our experimental data are consistent with the fact that commercially available Endo-F3 prefers core-fucosylated complex N-glycans and hydrolyzes them hundreds of times faster than it does the corresponding non-fucosylated N-glycans (65). Examination of the pH dependence of the Endo-F3-catalyzed hydrolysis using SGP as the substrate showed that the optimal pH for Endo-F3-catalyzed hydrolysis was around 4.5 at 37 °C, which is in good agreement with previous reports of the naturally isolated Endo-F3 (65).

**Generation and Characterization of Glycosynthase Mutants of Endo-F3**—We have previously generated glycosynthases from endoglycosidases of both the GH85 and GH18 family by site-directed mutation at a key residue that is responsible for promoting the formation of the oxazolinium ion intermediate during hydrolysis, which proceeds in a substrate-assisted mechanism (29, 30, 32–34). These include a key asparagine residue for the GH85 endoglycosidases Endo-A (Asn-171), Endo-M (Asn-275), and Endo-D (Asn-322) or a key aspartic acid residue for the GH18 family endoglycosidase Endo-S (Asp-233). A sequence alignment of Endo-F3 and Endo-S revealed that the Asp-165 of Endo-F3 was the residue equivalent to the Asp-233 of Endo-S essential for promoting oxazolinium ion formation in hydrolysis (Fig. 2). The crystal structure of Endo-F3 also suggested that the Asp-165 was critical for the hydrolysis (66). Thus, we generated two mutants, D165A and D165Q, using site-directed mutagenesis to create potential glycosynthase mutants of Endo-F3. The two mutants were also expressed as soluble proteins in the pET22b-CPD vector and produced similar yields as the wild type Endo-F3 enzyme. We then tested the potential hydrolytic activity of the Endo-F3 mutants using rituximab as the substrate under the conditions used to confirm the hydrolytic activity of Endo-F3 WT. Under the same conditions, the Endo-F3 mutants D165A and D165Q had no detectable hydrolytic activity on rituximab after 4 h when the reaction was monitored by LC-MS analysis. This data verify that the mutation eliminates the hydrolytic activity.

We have previously discovered a transglycosylation activity of wild type Endo-F3 using SCTox (1) as the donor substrate and Fmoc-Asn(Fucα1,6GlcNAc)-OH (core-fucosylated) (2) or Fmoc-Asn(GlcNAc)-OH (3) as the acceptor substrates (56). In this study, the same reactions were applied to test the transglycosylation activity of the Endo-F3 mutants using rituximab as the substrate under the conditions used to confirm the hydrolytic activity of Endo-F3 WT. Under the same conditions, the Endo-F3 mutants D165A and D165Q showed apparent transglycosylation activity with the core-fucosylated GlcNAc-Asn acceptor (2) to give the transglycosylation product (4) (Fig. 3B), which was identical to the authentic compound characterized previously by ESI-MS (56). Interestingly, no transglycosylation was detected when the non-fucosylated GlcNAc-Asn (3) was used as the substrate (Fig. 3). The results clearly indicate that the Endo-F3 glycosynthase mutants are highly specific and active for α1,6-fucosylated GlcNAc acceptors for transglycosylation. In contrast to the wild type Endo-F3, which had transglycosylation activity but gradually hydrolyzed the product, the D165A and D165Q mutants were able to use the sugar oxazolinone for transglycosylation without product hydrolysis, thus confirming them as typical glycosynthases. We also observed that the Endo-F3 D165A mutant was superior to the D165Q mutant in glycosynthase activity (Fig. 3B). Under the conditions, the Endo-F3 D165A-catalyzed reaction could reach 100% transglycosylation quickly without product hydrolysis, as monitored by reverse-phase HPLC and confirmed with ESI-MS analysis.

**Semisynthesis of Asialo Triantennary Complex Type Glycan Oxazoline**—Previous studies have shown that Endo-F3 can efficiently cleave fucosylated triantennary complex type N-glycans (65). The potent hydrolytic activity on branched complex N-glycans led us to investigate whether the glycosynthase mutants derived from Endo-F3 are able to transfer triantennary N-glycans. For this purpose, we carried out a semisynthesis of SCTox starting from the isolation of the N-glycans from bovine fetuin, a natural glycoprotein that carries sialylated bi- and triantennary N-glycans. The synthetic scheme was summarized in Fig. 4. Previously, the components and structures of N-glycans from bovine fetuin have been well characterized by chromatographic separation, HPAEC-PAD analysis, and detailed NMR studies (67–70). The majority (>80%) of the N-glycans were triantennary N-glycans with varied degrees of α2,6- and/or α2,3-sialylations at the terminus. For a relatively large scale preparation, we first purified bovine fetuin on the gram scale from fetal bovine serum using a previously reported method with some modifications (58). The N-glycans were released from bovine fetuin using Endo-F3 and were partially purified by acetone precipitation and extraction with 60% methanol (60, 71). The crude N-glycans showed multiple peaks on HPAEC-PAD with four major peaks as the triantennary complex type N-glycans carrying 2–3 sialic acids in isomeric α2,3- and α2,6-linkages (Fig. 5A). After desialating on a Sephadex G-25 column and then further purification by ion exchange chromatography using a HiTrap DEAE FF column, fractions that contained the triasialylated triantennary glycans were desalted using a Sephadex G-25 column giving two major triasialylated triantennary complex type N-glycan isomers differing only in the linkage of the sialic acid on the 6'-mannose arm (5) (Fig. 5B). The assignment of the peaks was based on a detailed HPAEC-PAD analysis of fetuin N-glycans reported previously (67, 69). The isomeric nature of the sialylated N-glycans was confirmed with ESI-MS analysis showing m/z species at 893.8 and 1340.01, respectively, corresponding to the [M + 3H]3+ and [M + 2H]2+ species (calculated M = 2677.39). Desialylation of 5 with sialidase gave TCT (6) as a single species with >95% purity (Fig. 5C). The only minor (<5%) contamination could be attributed to a triantennary N-glycan isomer that contains an isomeric α1,3-galacto-
sidic linkage, on the basis of the assignment reported previously (67–69). ESI-MS analysis confirmed the cleavage of the sialic acids, and the observed single species at \( m/z \) 902.46 corresponding to \([M + 2H]^{2+}\) is in good agreement with the calculated molecular mass \( (M = 1803.64)\). The triantennary N-glycan (6) was converted into the corresponding glycan oxazolines.
in a single step by treatment with an excess of 2-chloro-1,3-dimethylimidazolinium chloride and triethylamine in water, following the procedures reported previously (37, 57, 72). The reaction was monitored by HPAEC-PAD, and the product formation was found to be essentially quantitative (Fig. 5D).

HPAEC-PAD analysis clearly showed a shift of 3 min in retention time as the oxazoline derivative eluted before the free sugar (Fig. 5D). The product was purified from the reaction mixture by gel filtration on a Sephadex G15 column. The identities of the triantennary N-glycan (6) and the corresponding glycan oxazoline (7) were further characterized by two-dimensional 1H/13C HSQC NMR analysis (Fig. 6). The NMR signal assignment (Fig. 6A) of glycan 6 was in good agreement with a similar triantennary derivative with two GlcNAc moieties at the reducing terminus (70), and the results further confirmed the homogeneity of the asialylated triantennary N-glycan (6). The two-dimensional 1H/13C HSQC spectrum (Fig. 6B) of glycan oxazoline 7 clearly showed the expected downfield shift of the reducing end anomeric proton to 6.1 ppm with a 1,2 coupling constant of 7.3 Hz, which is a signature for the anomeric proton of sugar oxazoline.

**Endo-F3 Mutant D165A Is Able to Transfer Triantennary N-Glycan to Form Triantennary Complex Type Glycopeptides**—With the synthetic triantennary complex glycan oxazoline in hand, we selected the CD52 glycopeptide, a small glycosylphosphatidylinositol-anchored glycoprotein found on the surface of mature lymphocytes and sperm cells, as a target to test the Endo-F3 mutants and glycosynthase mutants from other endoglycosidases. We performed the transglycosylation with Endo-F3 D165A using the TCTox (7) as the donor and the Fuco1,6GlcNAc-CD52 (8) as the acceptor substrate, the synthesis of which was described previously (56). The reaction progress was monitored by HPLC analysis, and the product was analyzed with LC-MS. We found that the Endo-F3 D165A mutant could efficiently use the triantennary glycan oxazoline (7) as substrate for transglycosylation to form the corresponding triantennary complex type CD52 glycopeptide (9) (Fig. 7).

HPLC monitoring showed an m/z species of 1129.80 corresponding to [M + 3H]3+ (calculated molecular mass, M = 3385.09). We found that a quantitative conversion of the Fuco1,6GlcNAc-CD52 (8) to the glycopeptide (9) could be achieved within 2 h when a 3-fold excess of the glycan oxazoline (7) was used, demonstrating the efficiency of the Endo-F3 D165A-catalyzed transglycosylation. We have also tested several glycosyntheses described previously, including the D233A mutant of Endo-S, the N322A and N322Q mutants of Endo-D, the N175Q mutant of Endo-M, and the N171A mutant of Endo-A (29, 30, 32–34). We found that none of those glycosyntheses were able to transfer triantennary complex glycans from the glycan oxazoline. Thus, the Endo-F3 mutants generated here represent the first endoglycosidase-based glycosynthases capable of transforming branched triantennary complex type N-glycans for complex glycopeptide synthesis.
The Endo-F3 Glycosynthase Mutant Enables the Glycosylation Remodeling of Intact Monoclonal Antibody—The ability of the Endo-F3-derived glycosynthases to transfer the triantennary glycan for the synthesis of complex CD52 glycopeptide encouraged us to test whether the Endo-F3 mutant would also be efficient at transferring triantennary N-glycans to intact antibodies. We have previously used mutant endoglycosidases to remodel the N-glycans on the Fc region of the monoclonal antibody rituximab using a pair of endoglycosidase/endoglycosylsynthase derived from Endo-S (33). The major Fc glycans of commercial rituximab are core-fucosylated biantennary complex type oligosaccharides carrying 0–2 galactose moieties named the G0F, G1F, and G2F glycoform, respectively. The general glycosylation remodeling approach is presented in Fig. 8, and the reactions and reaction products were assessed by LC-MS analysis (Fig. 9).

Treatment of rituximab (10) with recombinant Endo-F3 resulted in complete deglycosylation of rituximab, as demonstrated by the conversion of the glycoform mixtures (G0F, G1F, and G2F) found in commercial rituximab (Fig. 9A) to the Fuca1,6GlcNAc-glycoform (11) of rituximab (Fig. 9B). The deglycosylated rituximab was purified away from the WT...
endoglycosidase and excess glycans by affinity chromatography with a protein A column to give the Fuc\(\alpha\)1,6GlcNAc-rituximab intermediate (11). We found that Endo-F3 D165A was able to efficiently transfer sialylated and asialylated biantennary \(N\)-glycans from the corresponding glycan oxazolines (1 and 13) to the Fuc\(\alpha\)1,6GlcNAc-rituximab acceptor (11) to form the respective new glycoforms of rituximab (12 and 14). Likewise, the Endo-F3 D165A mutant was also efficient to glycosylate the Fuc\(\alpha\)1,6GlcNAc-rituximab (11) using the triantennary glycan oxazoline (7) as the donor substrate to give the triantennary complex glycoform of rituximab (15). The reaction could be readily pushed to completion when a 40–50 molar equivalent (i.e. 20–25 molar equivalent per monomeric Fc domain) of the glycan oxazoline was added in two portions. The reaction yield was estimated by LC-MS analysis to be over 95% because almost no starting material was detected, which confirmed the completion of the transglycosylation. The rituximab remodeling progress was first monitored by SDS-PAGE analysis. In all cases, the products showed a single band for the heavy chain that was \(\sim 2\) kDa larger than the heavy chain of the Fuc\(\alpha\)1,6GlcNAc-rituximab, whereas the light chain was a single band with the same size before and after the reactions. These data suggest that a single \(N\)-glycan was attached to each of the heavy chain of the intact antibody without modification on the light chain. LC-MS analysis of the transglycosylation products (12 and 14) carrying biantennary \(N\)-glycans revealed that the heavy chain of 12 and 14 appeared as a single species at 51,412 and 50,835 (deconvolution data), respectively, which are in good agreement with the calculated molecular mass \((M = 51,421\) and 50,839 Da) for the heavy chains carrying a sialylated or asialylated biantennary \(N\)-glycan (with core fucose), respectively (Fig. 9, C and D). In the case of the triantennary glycoform (15), the deconvolution data of the ESI-MS revealed a single peak at 51,204 for the heavy chain of 15, which matches well the calculated molecular mass of the heavy chain carrying a single triantennary \(N\)-glycan \((M = 51,199\) Da) (Fig. 9E).

To further confirm that the \(N\)-glycan was attached specifically to the Asn-297 \(N\)-glycosylation site of the Fc domain, instead of other sites of the polypeptide backbone that might occur by non-enzymatic reactions, we treated the transglycosylation products with PNGase F and examined the protein portion and \(N\)-glycan portion by mass spectrometry analysis. PNGase F was highly specific and could release the \(N\)-glycans only when they were attached to the Asn side chain in an \(N\)-glycosylamide linkage at the conserved glycosylation site in the glycoproteins. LC-MS analysis of the heavy chain of the PNGase F-treated transglycosylation product (12) gave a species of 49,066 (deconvolution data), which matches well with
the calculated molecular mass of the polypeptide backbone of the heavy chain without any additional modifications (\(M = 49,069\) Da) (Fig. 9F). On the other hand, the light chain of the transglycosylation product (12) appeared as a single species at 23,038, which matches the calculated molecular mass of the light chain of rituximab (\(M = 23,039\) Da) (Fig. 9G). Taken together, these results clearly indicated that a single sialylated biantennary N-glycan was conjugated to the antibody heavy chain, and the intact N-glycan was attached at the conserved N-glycosylation site without any non-enzymatic conjugation of N-glycans. We also tested the glycosynthase mutant, Endo-S D233A, which was previously shown to be capable of transferring biantennary N-glycan to intact antibody acceptor. We found that Endo-S D233A was unable to transfer the triantennary glycan oxazoline (7) to rituximab because no transglycosylation product was detected even after a prolonged incubation (over 5 h).

**Binding of the Glycoengineered Rituximab to the C-type Lectin Galectin-3**—The affinity of the remodeled glycoforms of rituximab for galectin-3 was examined by SPR analysis. Galectin-3 was immobilized on the CM5 chip, and the various rituximab glycoforms were injected as analytes, following our previous procedures (73). The commercially available rituximab showed no detectable binding under the concentrations tested (Fig. 10A). However, both the biantennary and triantennary glycoforms, G2F and G3F, showed strong binding to galectin-3 (Fig. 10, B and C). The \(K_D\) values for the bi- and triantennary glycoforms were estimated to be 130 and 64 nM, respectively, indicating that the terminal galactosylated triantennary glycoform of antibody has higher affinity for galectin-3 than that of the terminal galactosylated biantennary antibody glycoform.

**Binding of the Glycoengineered Rituximab to the FcγRIIIa Receptor (FcγRIIIa)**—The affinity of the remodeled glycoforms of rituximab for FcγRIIIa was examined by SPR analysis. Protein A was nonspecifically bound to the CM5 chip and used to capture the various rituximab glycoforms. After capture of antibody glycoforms, the FcγRIIIa was injected as the analyte to obtain the SPR responses (Fig. 11). Fitting the data with the 1:1 binding model did not give reliable kinetic parameters. Then the data were analyzed by using the steady state fitting (Fig. 11). The \(K_D\) values for the binding of FcγRIIIa to the commercial rituximab and the G2F and the G3F glycoforms were estimated to be 245 ± 1, 162 ± 5, and 168 ± 8 nM, respectively. The G2F and G3F glycoforms showed similar affinity for FcγRIIIa, but their affinity for the FcγRIIIa is slightly higher than that of the commercially available rituximab.

**Discussion**

In this study, we have reported a successful and high yield expression of soluble recombinant Endo-F3 as a CPD fusion protein, along with the generation and expression of glycosynthase mutants that possess potent transglycosylation activity with glycan oxazolines but are devoid of product hydrolysis activity. We have also established an efficient semisynthesis of triantennary complex type N-glycan and the corresponding glycan oxazoline, which will be useful substrates for assessment of new glycosynthase mutants. It should be mentioned that previous attempts to express soluble Endo-F3 have met with little success, because of inclusion body formation (61, 62). The new Endo-F3 CPD construct makes it possible to express soluble and active Endo-F3 and its mutants on a large scale with high expression yield.
We have found that the two glycosynthase mutants generated, D165A and D165Q, are highly specific for fucosylated GlcNAc acceptor. The significant difference in transglycosylation activity with the core-fucosylated and non-fucosylated GlcNAc acceptors appears consistent with the known substrate specificity of Endo-F3 for N-glycan hydrolysis. It was reported previously that wild type Endo-F3 hydrolyzed core-fucosylated glycopeptides more than 300-fold faster than the corresponding non-fucosylated glycopeptide substrates (65). An important discovery in this study is that the Endo-F3 glycosynthase mutants are able to transfer triantennary complex type N-glycan to fucosylated GlcNAc-peptides and deglycosylated monoclonal antibodies, which could not be achieved by previously reported glycosynthases, such as those derived from Endo-A, Endo-M, Endo-D, and Endo-S. This highly convergent chemoenzymatic assembly of complex glycopeptides provides an alternative to pure chemical synthesis of biologically important glycopeptides, such as tumor-associated glycopeptide antigens useful for development of vaccines and diagnostic tools (74). We also measured the affinity of the terminal fully galactosylated triantennary antibody glycoform with galectin-3, a C-type lectin. Galectin-3 recognizes β-galactoside-terminating glycoconjugates in biological systems and plays important roles in a number of biological processes, including cell adhesion, autoimmunity, and cancer metastasis (78). The fact that the new triantennary complex type glycoform demonstrates much higher affinity for galectin-3 than that of the commercial rituximab suggests that the glycosylation-remodeled antibody may confer novel properties to the antibodies in vivo. Moreover, a
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recent report has demonstrated that IgGs with terminal galactose residues in the Fc glycosylation interact with Dectin-1 and Fc receptor to elicit an anti-inflammatory response (79). It would be interesting to test whether the newly obtained, fully galactosylated Fc glycoforms of antibodies will show enhanced anti-inflammatory activities in animal models.

In summary, the Endo-F3 mutants (D165A and D165Q) described in this study represent the first endoglycosidase-based glycosynthases capable of accepting triantennary complex type N-glycans for transglycosylation. The ability of the Endo-F3 mutants to transfer triantennary complex type N-glycans to α1,6-fucosylated GlcNAc-polypeptides and deglycosylated intact antibodies significantly expands the scope of the chemoenzymatic method, which could not be achieved by the previously reported glycosynthases, such as the Endo-S and Endo-M mutants. It is expected that the new Endo-F3 glycosynthases will find useful applications for convergent chemoenzymatic synthesis of core-fucosylated complex type glycans to accept triantennary complex type N-glycans for transglycosylation. The ability of the newly obtained, fully galactosylated Fc glycoforms of antibodies will show enhanced antitumour vaccines from mucin glycopeptide antigens. Chem. Soc. Rev. 42, 4421–4442

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References
1. Weerapana, E., and Imperiali, B. (2006) Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems. Glycobiology 16, 91R–101R
2. Dwek, R. A. (1996) Glycobiology: toward understanding the function of sugars. Chem. Rev. 96, 683–720
3. Helenius, A., and Aebi, M. (2001) Intracellular functions of N-linked glycans. Science 291, 2364–2369
4. Dwek, R. A., Butters, T. D., Platt, F. M., and Zitzmann, N. (2002) Targeting glycosylation as a therapeutic approach. Nat. Rev. Drug Discov. 1, 65–75
5. Haltiwanger, R. S., and Lowe, J. B. (2004) Role of glycosylation in development. Annu. Rev. Biochem. 73, 491–537
6. Dube, D. H., and Bertozzi, C. R. (2005) Glycans in cancer and inflammation: potential for therapeutics and diagnostics. Nat. Rev. Drug Discov. 4, 477–488
7. Walsh, G., and Jefferis, R. (2006) Post-translational modifications in the context of therapeutic proteins. Nat. Biotechnol. 24, 1241–1252
8. Taniguchi, N., and Kizuka, Y. (2015) Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. Adv. Cancer Res. 126, 11–51
9. Jenkins, N., Parekh, R. B., and James, D. C. (1996) Getting the glycosylation right: implications for the biotechnology industry. Nat. Biotechnol. 14, 975–981
10. Arnold, J. N., Wormald, M. R., Sim, R. B., Rudd, P. M., and Dwek, R. A. (2007) The impact of glycosylation on the biological function and structure of human immunoglobulins. Annu. Rev. Immunol. 25, 21–50
11. Jefferis, R. (2009) Glycosylation as a strategy to improve antibody-based therapeutics. Nat. Rev. Drug Discov. 8, 226–234
12. Dalziel, M., Crispin, M., Scanlan, C. N., Zitzmann, N., and Dwek, R. A. (2014) Emerging principles for the therapeutic exploitation of glycosylation. Science 343, 1235681
13. Buskas, T., Ingle, S., and Boons, G. J. (2006) Glycopeptides as versatile tools for glycobiology. Glycobiology 16, 113R–136R
14. Gamblin, D. P., Scanlan, E. M., and Davis, B. G. (2009) Glycoprotein synthesis: an update. Chem. Rev. 109, 131–163
15. Yuan, Y., Chen, J., Wan, Q., Wilson, R. M., and Danishefsky, S. J. (2010) Toward fully synthetic, homogeneous glycoproteins: advances in chemical ligation. Biopolymers 94, 373–384
16. Gaidzik, N., Westerlind, U., and Kunz, H. (2013) The development of synthetic antitumour vaccines from mucin glycopeptide antigens. Chem. Soc. Rev. 42, 4421–4442
17. Unverzagt, C., and Kajihara, Y. (2013) Chemical assembly of N-glycoproteins: a refined toolbox to address a ubiquitous posttranslational modification. Chem. Soc. Rev. 42, 4408–4420
18. Hang, H. C., and Bertozzi, C. R. (2001) Chemoselective approaches to glycoprotein assembly. Acc. Chem. Res. 34, 727–736
19. Chalker, J. M., Bernardes, G. J., and Davis, B. G. (2011) A “tag-and-modify” approach to site-selective protein modification. Acc. Chem. Res. 44, 730–741
20. Wang, L. X. (2008) Chemoenzymatic synthesis of glycopolptides and glycoproteins through endoglycosidase-catalyzed transglycosylation. Carbohydr. Res. 343, 1509–1522
21. Rich, J. R., and Withers, S. G. (2009) Emerging methods for the production of homogeneous human glycoproteins. Nat. Chem. Biol. 5, 206–215
22. Schmalz, R. M., Hanson, S. R., and Wong, C. H. (2011) Enzymes in the synthesis of glycoconjugates. Chem. Rev. 111, 4259–4307
23. Wang, L. X., and Lomino, J. V. (2012) Emerging technologies for making glycan-defined glycoproteins. ACS Chem. Biol. 7, 110–122
24. Wang, L. X., and Amin, M. N. (2014) Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. Chem. Biol. 21, 51–66
25. Stanley, P. (1992) Glycosylation engineering. Glycobiology 2, 99–107
26. Hamilton, S. R., and Gerngross, T. U. (2007) Glycosylation engineering in yeast: the advent of fully humanized yeast. Curr. Opin. Biotechnol. 18, 387–392
27. Jacobs, P. P., Geyens, S., Vervecken, W., Contreras, R., and Callewaert, N. (2009) Engineering complex-type N-glycosylation in Pichia pastoris using GlycoSwitch technology. Nat. Protoc. 4, 58–70
28. Castillo, A., and Steinkeller, H. (2012) Glyco-engineering in plants to produce human-like N-glycan structures. Biotechnol. J. 7, 1088–1098
29. Umekawa, M., Huang, W., Li, B., Fujita, K., Ashida, H., Wang, L. X., and Yamamoto, K. (2008) Mutants of Mucor hiemalis endo-β-N-acetylglucosaminidase show enhanced transglycosylation and glycosylase-like activities. J. Biol. Chem. 283, 4469–4479
30. Umekawa, M., Li, C., Higashiyama, T., Huang, W., Ashida, H., Yamamoto, K., and Wang, L. X. (2010) Efficient glycosynthetic mutant derived from Mucor hiemalis endo-β-N-acetylglucosaminidase capable of transferring oligosaccharide from both sugar oxazoline and natural N-glycan. J. Biol. Chem. 285, 511–521
31. Umekawa, M., Higashiyama, T., Koga, Y., Tanaka, T., Noguchi, M., Kobayashi, A., Shoda, S., Huang, W., Wang, L. X., Ashida, H., and Yamamoto, K. (2010) Efficient transfer of siaio-oligosaccharide onto proteins by combined use of a glycosylase-like mutant of Mucor hiemalis endoglycosidase-d and synthetic siaio-complex-type sugar oxazoline. Biochim. Biophys. Acta. 1800, 1203–1209
32. Huang, W., Li, C., Li, B., Umekawa, M., Yamamoto, K., Zhang, X., and Wang, L. X. (2009) Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-glycoproteins carrying intact natural N-glycans. J. Am. Chem. Soc. 131, 2214–2223
33. Huang, W., Giddens, J., Fan, S. Q., Toonstra, C., and Wang, L. X. (2012) Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. J. Am. Chem. Soc. 134, 12308–12318
34. Fan, S. Q., Huang, W., and Wang, L. X. (2012) Remarkable transglycosylation activity of glycosynthetic mutants of Endo-D, an endo-beta-N-acetylglucosaminidase from Streptococcus pneumoniae. J. Biol. Chem. 287, 11272–11281
Novel Endo-F3 Glycosyntheses for Glycoprotein Synthesis

35. Amin, M. N., McLellan, J. S., Huang, W., Orwenyo, J., Burton, D. R., Koff, W. C., Kwong, P. D., and Wang, L. X. (2013) Synthetic glycopeptides reveal the glycan specificity of HIV-neutralizing antibodies. *Nat. Chem. Biol.* 9, 521–526

36. Amin, M. N., Huang, W., Mizanur, R. M., and Wang, L. X. (2011) Convergent synthesis of homogeneous Glc(1)Man(9)GlcNAc(2)-protein and derivatives as ligands of molecular chaperons in protein quality control. *J. Am. Chem. Soc.* 133, 14404–14417

37. Huang, W., Zhang, X., Ju, T., Cummings, R. D., and Wang, L. X. (2010) Expeditious chemoenzymatic synthesis of CD52 glycopeptide antigens. *Org. Biomol. Chem.* 8, 5224–5233

38. Hojo, H., Tanaka, H., Hagiwara, M., Asahina, Y., Ueki, A., Katayama, H., Nakahara, Y., Yoneshige, A., Matsuda, J., Ito, Y., and Nakahara, Y. (2012) Chemoenzymatic synthesis of hydrophobic glycoprotein: synthesis of N-pasposi Ccarrying complex-type carbohydrate. *J. Org. Chem.* 77, 9437–9446

39. Lin, C. W., Tsai, M. H., Li, S. T., Tsai, T. I., Chu, K. C., Liu, Y. C., Lai, M. Y., Wu, C. Y., Tseng, Y. C., Shvivate, S. S., Wang, C. H., Chao, P. W., Yang, S. Y., Shih, H. W., Zeng, Y. F., et al. (2015) A common glycan structure on immunoglobulin G for enhancement of effector functions. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10611–10616

40. Karogochi, M., Mori, M., Osumi, K., Tojino, M., Sugawara, S., Takashima, S., Hirose, Y., Tsukimura, W., Mizuno, M., Amano, J., Matsuda, A., Tohmi, M., Takayanagi, A., Shoda, S., and Shirai, T. (2015) Glycoengineered fucose and bisecting GlcNAc, the direct modifiers of the pancreatic cancer. *Am. J. Pathol.* 186, 388–400

51. Wang, P., Zhu, J., Yuan, Y., and Danishefsky, S. J. (2009) Total synthesis of the 2,6-sialylated immunoglobin G glycopeptide fragment in homogeneous form. *J. Am. Chem. Soc.* 131, 16669–16671

52. Li, L., Liu, Y., Ma, C., Qu, J., Calderon, A. D., Wu, B., Wei, N., Wang, X., Guo, Y., Xiao, Z., Song, J., Sugiarito, G., Li, Y., Yu, H., Chen, X., and Wang, P. G. (2015) Efficient chemoenzymatic synthesis of an N-glycan isomer library. *Chem. Sci.* 6, 5652–5661

53. Trimbile, R. B., and Tarentino, A. L. (1991) Identification of distinct endoglycosidase (endo) activities in *Flavobacterium meningosepticum*: endo F1, endo F2, and endo F3: endo F1 and endo H hydrolyze only high manose and hybrid glycans. *J. Biol. Chem.* 266, 1646–1651

54. Tarentino, A. L., Quinones, G., Changchien, L. M., and Plummer, T. H., Jr. (1993) Multiple endoglycosidase F activities expressed by *Flavobacterium meningosepticum* endoglycosidases F2 and F3: molecular cloning, primary sequence, and enzyme expression. *J. Biol. Chem.* 268, 9702–9708

55. Kim, K. K., Kim, M. K., Lim, J. H., Park, H. Y., and Lee, S. T. (2005) Transfer of Chryseobacterium meningosepticum and Chryseobacterium miriocola to Elizabethkingia gen. nov. as Elizabethkingia meningoseptica comb. nov., and Elizabethkingia miriocola comb. nov. *Int. J. Syst. Evol. Microbiol.* 55, 1287–1293

56. Huang, W., Li, J., and Wang, L. X. (2011) Unusual transglycosylation activity of *Flavobacterium meningosepticum* endoglycosidases convergent chemoenzymatic synthesis of core fusosylated complex N-glycopeptides. *ChemBioChem* 12, 932–941

57. Huang, W., Yang, Q., Umekawa, M., Yamamoto, K., and Wang, L. X. (2010) Arthrobacter endo-β-N-acetylgalactosaminidase shows transglycosylation activity on complex-type N-glycan oxazolines: one-pot conversion of ribonuclease B to sialylated ribonuclease C. *ChemBioChem* 11, 1350–1355

58. Spiro, R. G. (1960) Studies on fetuin, a glycoprotein of fetal serum. I. Isolation, chemical composition, and physiochemical properties. *J. Biol. Chem.* 235, 2860–2869

59. Mosesson, M. W., and Sherry, S. (1966) The preparation and properties of human fibrinogen of relatively high solubility. *Biochemistry* 5, 2829–2835

60. Verostek, M. F., Lubowski, C., and Trimble, R. B. (2000) Selective precipitation/extraction of released N-glycans following large-scale enzymatic deglycosylation of glycoproteins. *Anal. Biochem.* 286, 111–122

61. Tarentino, A. L., Quinones, G., and Plummer, T. H., Jr. (1995) Overexpression and purification of non-glycosylated recombinant endo-β-N-acetylgalactosaminidase F3. *Glycobiology* 5, 599–601

62. Tarentino, A., Plummer, T., Jr., and Quinones, G. (1999) An improved amplification system for the production of Endo F3. *Glycobiology* 9, iii

63. Shen, A., Lupardus, P. J., Morell, M., Ponder, E. L., Sadaghiani, A. M., Garcia, K. C., and Bogoy, M. (2009) Simplified, enhanced protein purification using an inducible, autoprocessing enzyme tag. *PLoS One* 4, e8119

64. Lomino, J. V., Tripathy, A., and Redinbo, M. R. (2011) Triggered *Mycobacterium tuberculosis* heparin-binding hemagglutinin adhesion folding and dimerization. *J. Bacteriol.* 193, 2089–2096

65. Plummer, T. H., Jr., Phelan, A. W., and Tarentino, A. L. (1996) Porcine fibrinogen glycopeptides: substrates for detecting endo-β-N-acetylgalactosaminidases F2 and F3(1). *Anal. Biochem.* 235, 98–101

66. Wadding, C. A., Plummer, T. H., Jr., Tarentino, A. L., and Van Roey, P. (2000) Structural basis for the substrate specificity of endo-β-N-acetylgalactosaminidase F3. *Biochemistry* 39, 7878–7885

67. Hardy, M. R., and Townsend, R. R. (1988) Separation of positional isomers of oligosaccharides and glycopeptides by high-performance anion-exchange chromatography with pulsed amperometric detection. *Proc. Natl. Acad. Sci. U.S.A.* 85, 3289–3293

68. Bendikan, S., Harris-Brandts, M., Mischnick, S. W., Carver, J. P., and Cumming, D. A. (1990) Separation of the complex asparagine linked oligosaccharides of the glycoprotein fetuin and elucidation of three triantennary structures having sialic acids linked only to galactose residues. *Biochemistry* 28, 6491–6499

69. Townsend, R. R., Hardy, M. R., Cumming, D. A., Carver, J. P., and Bendikan, B. (1989) Separation of branched sialylated oligosaccharides using high-pH anion-exchange chromatography with pulsed amperometric detection. *Anal. Biochem.* 182, 1–8

70. Sato, H., Fukae, K., and Kajihara, Y. (2008) 2D selective-TOCSY-DQF-COSY and HSQC-TOSY NMR experiments for assignment of a homogeneous asparagine-linked triantennary complex type undecaaccharide. *Carbohydr. Res.* 343, 1333–1345
71. Green, E. D., Adelt, G., Baenziger, J. U., Wilson, S., and Van Halbeek, H. (1988) The asparagine-linked oligosaccharides on bovine fetuin: structural analysis of N-glycanase-released oligosaccharides by 500-megahertz $^1$H NMR spectroscopy. *J. Biol. Chem.* **263**, 18253–18268

72. Noguchi, M., Tanaka, T., Gyakushi, H., Kobayashi, A., and Shoda, S. (2009) Efficient synthesis of sugar oxazolines from unprotected N-acetyl-2-amino sugars by using chloroformamidinium reagent in water. *J. Org. Chem.* **74**, 2210–2212

73. Wang, H., Huang, W., Orwenyo, J., Banerjee, A., Vasta, G. R., and Wang, L. X. (2013) Design and synthesis of glycoprotein-based multivalent glycopoligands for influenza hemagglutinin and human galectin-3. *Bioorg. Med. Chem.* **21**, 2037–2044

74. Walczak, M. A., and Danishefsky, S. J. (2012) Solving the convergence problem in the synthesis of triantennary N-glycan relevant to prostate-specific membrane antigen (PSMA). *J. Am. Chem. Soc.* **134**, 16430–16433

75. Castilho, A., Neumann, L., Gattinger, P., Strasser, R., Vorauer-Uhl, K., Sterovsky, T., Altmann, F., and Steinkellner, H. (2013) Generation of biologically active multi-sialylated recombinant human EPOFc in plants. *PLoS One* **8**, e54836

76. Raju, T. S., Briggs, J. B., Borge, S. M., and Jones, A. J. (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. *Glycobiology* **10**, 477–486

77. Rose, R. J., van Berkel, P. H., van den Bremer, E. T., Labrijn, A. F., Vink, T., Schuurman, J., Heck, A. J., and Parren, P. W. (2013) Mutation of Y407 in the CH3 domain dramatically alters glycosylation and structure of human IgG. *MAbs* **5**, 219–228

78. Funasaka, T., Raz, A., and Nangia-Makker, P. (2014) Galectin-3 in angiogenesis and metastasis. *Glycobiology* **24**, 886–891

79. Karsten, C. M., Pandey, M. K., Figge, J., Kilchenstein, R., Taylor, P. R., Rosas, M., McDonald, J. U., Orr, S. J., Berger, M., Petzold, D., Blanchard, V., Winkler, A., Hess, C., Reid, D. M., Majoul, I. V., *et al.* (2012) Anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcgammaRIIB and dectin-1. *Nat. Med.* **18**, 1401–1406