Remarkable Transglycosylation Activity of Glycosynthase Mutants of Endo-D, an Endo-β-N-acetylgalactosaminidase from *Streptococcus pneumoniae*\(^*\)

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Background: Endo-D is an endoglycosidase that can deglycosylate N-glycan core from IgG antibodies.

Results: Endo-D showed distinct activities toward core-fucosylated and nonfucosylated substrates, and novel mutants were generated that demonstrated remarkable transglycosylation activity.

Conclusion: The new discovery expands the repertoire of endoenzymes for glycoprotein research.

Significance: This study reveals interesting substrate specificity of Endo-D and provides new enzymatic tools for glycosylation engineering of glycoproteins such as IgG-Fc.

**Endo-β-N-acetylgalactosaminidase from *Streptococcus pneumoniae* (Endo-D) is an endoglycosidase capable of hydrolyzing the Fc N-glycan of intact IgG antibodies after sequential removal of the sialic acid, galactose, and internal GlcNAc residues in the N-glycan.** Endo-D also possesses transglycosylation activity with sugar oxazoline as the donor substrate, but the transglycosylation yield is low due to enzymatic hydrolysis of the donor substrate and the product. We report here our study on the hydrolytic and transglycosylation activity of recombinant Endo-D and its selected mutants. We found that Endo-D preferred core-fucosylated N-glycan for hydrolysis but favored nonfucosylated GlcNAc acceptor for transglycosylation. Several mutants showed significantly enhanced transglycosylation efficiency over the wild type enzyme. Two mutants (N322Q and N322A) were identified as typical glycosynthases that demonstrated remarkable transglycosylation activity with only marginal or no product hydrolysis activity. Kinetic studies revealed that the N332Q and N322A glycosynthases had much higher catalytic efficiency for glycosylating the nonfucosylated GlcNAc acceptor. In comparison, the N322Q was much more efficient than N322A for transglycosylation. However, N332Q and N322A could not take more complex N-glycan oxazoline as substrate for transglycosylation, indicating their strict substrate specificity. The usefulness of the N332Q glycosynthase was exemplified by its application for efficient glycosylation remodeling of IgG-Fc domain.

Endo-β-N-acetylgalactosaminidases (ENGases)\(^3\) are a class of glycoside hydrolases that hydrolyze the β-1,4-glycosidic bond in the N,N'-diacetyldichitobiose core of N-glycans. The deglycosylation property of ENGases has been frequently used for structural and functional studies of glycoproteins. ENGases are classified into two classes of the glycoside hydrolase (GH) families in the CAZY database, GH18 and GH85. The commonly used bacterial endo-β-N-acetylgalactosaminidases, such as Endo-H from *Streptomyces plicatus* (1) and Endo-F₁, Endo-F₂, and Endo-F₃ from *Flavobacterium meningosepticum* (2, 3), belong to the GH18 family. In contrast, the GH85 family includes ENGases from both prokaryotes and eukaryotes, including Endo-M from *Mucor hiemalis* (4, 5), Endo-A from *Arthrobacter protophormiae* (6), Endo-D from *Streptococcus pneumoniae* (7), and Endo-CE from *Caenorhabditis elegans* (8). In addition to the hydrolytic activity, some of the ENGases have been shown to possess transglycosylation activity, capable of transferring the released N-glycan to an alcoholic acceptor such as an N-acetylgalactosamine (GlcNAc) moiety to reconstitute the natural β-1,4-glycosidic linkage. The transglycosylation activity of ENGases has attracted much attention in recent years for chemoenzymatic synthesis of oligosaccharides, glycopeptides, and glycoproteins (9, 10).

Structural and mechanistic studies on Endo-H (11), Endo-F₁ (12), Endo-F₃ (13), Endo-A (14, 15), and Endo-D (16) suggest that the ENGase-catalyzed N-glycan hydrolysis follows a substrate-assisted mechanism. In this mechanism, a general acid/base residue (Asp or Glu) first acts as a general acid to protonate the glycosidic oxygen. Upon activation, the 2-acetamide group of the (−)GlcNAc in the substrate acts as a nucleophile to attack the anomeric center, resulting in the breakdown of the glycosidic bond with simultaneous formation of an oxazolinium ion intermediate. The oxazolinium intermediate then undergoes hydrolysis or transglycosylation via its reaction with a water molecule or an alcoholic acceptor activated by the general acid/base residue. These structural studies also identified

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\(^{\[\text{3}\]}\)The abbreviations used are: ENGase, endo-β-N-acetylgalactosaminidase; Endo-D, endo-β-N-acetylgalactosaminidase from *S. pneumoniae*; GH, glycoside hydrolase; CT, complex type; RP-HPLC, reverse-phase high performance liquid chromatography; ESI-MS, electron spray ionization-mass spectrometry; FMoc, N-(9-fluorenylmethoxycarbonyl); DMSO, dimethyl sulfoxide; a.a., amino acids.
another important residue, which is located at 1 or 2 amino acid residues upstream from the general acid/base catalytic residue. This key residue was shown to be an Asp residue for the GH18 ENGases (Endo-H, Endo-F$_4$, and Endo-F$_3$) or an Asn residue for the GH85 ENGases (Endo-A, Endo-M, and Endo-D), which was required for the proper orientation of the acetamide group to promote the oxazolinium ion formation. The essential role of this residue for hydrolysis was confirmed by the fact that mutation of this residue abolished the hydrolytic activity of ENGases (11–16). These mechanistic and mutagenesis studies laid the basis for exploring synthetic sugar oxazolines as donor substrates for transglycosylation, which resulted in significant enhancement of the transglycosylation efficiency for glycopeptide and glycoprotein synthesis (17–22). Moreover, it was further demonstrated that novel glycosynthases could be generated by site-directed mutation at the critical Asn residue that promotes oxazolinium intermediate formation in hydrolysis (Asn-175 in Endo-M and Asn-171 in Endo-A). The resulting mutants such as EndoM-N175Q and EndoA-N171Q were able to take the activated sugar oxazolines for transglycosylation with marginal or abolished hydrolytic activity on the transglycosylation product (23–29). These discoveries open a new avenue to glycoprotein synthesis and glycosylation remodeling. Nevertheless, different ENGases have distinct substrate specificity and limitations. For example, although Endo-A is usually specific for high mannose type N-glycans, and Endo-M works on biantenary complex type N-glycans, neither Endo-A nor Endo-M is able to act on core-fucosylated N-glycans. New glycosynthases with different substrate specificity are required to expand the synthetic repertoire of this class of enzymes.

Endo-D from *S. pneumoniae* belongs to the glycoside hydrolase family 85 (GH85). However, in contrast to Endo-A and Endo-M that belong to the same family, Endo-D is able to hydrolyze fucosylated N-glycan core. Fairbanks and co-workers (30) first reported that Endo-D possessed transglycosylation activity, capable of using Man$_3$GlcNAc oxazoline as donor substrate to glycosylate a GlcNAc acceptor, but the transglycosylation efficiency was low. Our subsequent study confirmed the results and further demonstrated that when a fucosylated GlcNAc acceptor was used, Endo-D gave only marginally detectable transglycosylation product (31). This observation might be due to the quick hydrolysis of the sugar oxazoline and/or the transglycosylation product by the wild type enzyme. In this study, we report an expanded study on the hydrolysis and transglycosylation activity of recombinant Endo-D and its selected mutants. Our studies indicate that Endo-D demonstrates significantly different activity toward core-fucosylated and nonfucosylated substrates for both hydrolysis and transglycosylation. Moreover, we have created novel glycosynthase mutants that show remarkably enhanced transglycosylation efficiency without product hydrolysis. The usefulness of the glycosynthase mutants in synthesis was demonstrated by its efficient application for glycosylation remodeling of IgG-Fc domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—The synthesis of Fmoc-Asn(Fucα1,6GlcNAc)-OH, Fmoc-Asn(GlcNAc)-OH, Fmoc-Asn(Man$_3$GlcNAc$_2$Fuc)-OH, and Fmoc-Asn (Man$_3$GlcNAc$_2$)-OH was described in our previous publication (31). Man$_3$GlcNAc-oxazoline was synthesized according to our previously reported procedure (18). Monoclonal antibody rituximab (rituxan, Genentech Inc., South San Francisco, CA) was purchased through Premium Health Services Inc. (Columbia, MD). Papain was purchased from Sigma-Aldrich. The endoglycosidase from *Streptococcus pyogenes* (Endo-S) was expressed and purified according to the reported procedure (32, 33).

**Cloning, Expression, and Purification of Endo-D in Escherichia coli**—The cDNA fragment encoding the Endo-D fragment (nucleotides 403–3141; amino acids 135–1047), here called Endo-D, was amplified by PCR from the genomic DNA of *S. pneumoniae* (ATCC number: BA.A.-334D-5). The forward primer was 5'-TATATACATATGGAGTCTA.A.ACCAGCA-GCAGA.A.GC-3', and the reverse primer was 5'-GCCGCG CTCCGATTCTCTGATCATTTTGGAG.A.CCG-3'. Ndel and XhoI site (underlined) were added to the forward and reverse primers, respectively. The cDNA fragment of a further truncated form (nucleotides 475–2471; amino acids 157–807) of Endo-D (called spGH85) was cloned following the previously reported procedure (16). Both of the amplified DNA fragments were cloned into pET28a (Novagen) after digestion with Ndel and XhoI. The constructed plasmids, pET28a-EndoD and pET28a-spGH85, respectively, were transformed into BL21 (DE3). The transformants were cultured in LB media supplemented with 50 μg/ml kanamycin. Cultures were grown at 37°C until the cells reached an absorbance of 0.5–0.8 at 600 nm. Then 0.5 mm isopropyl β-D-1-thiogalactopyranoside was added to the culture to induce protein overproduction. After further incubation at 25°C for 8 h, the cells were harvested by centrifugation. The cell pellets were suspended in a sodium phosphate buffer (50 mM, pH 7.0) with lysozyme before sonication. After sonication and centrifugation, the supernatant from the cell lysis was applied onto an Ni$^{2+}$-immobilized HisTrap HP column (GE Healthcare). The column was washed with 50 mM imidazole and then eluted with 200 mM imidazole in a buffer containing 0.5 M NaCl and 0.1 M sodium phosphate (pH 7.4). The eluent was desalted and concentrated by Amicon Ultra filtration (10 kDa, Millipore, Billerica, MA). The homogeneity of the recombinant Endo-D and spGH85 was confirmed by SDS-PAGE with Coomassie Brilliant Blue staining. The protein concentration was quantified using the Bradford assay protocol with bovine serum albumin (BSA) as standard.

**Site-directed Mutagenesis of Endo-D**—The selected mutants (N322A, N322Q, E324Q, Y360F, and H371W) were generated using the GENEART site-directed mutagenesis kit (Invitrogen) per the manufacturer’s directions. The pET28a-EndoD plasmid encoding the Endo-D gene (nucleotides 403–3141; amino acids 135–1047) was used as the template, and *L. lactis* polymerase (Takara) was used for PCR. Mutations were confirmed by DNA sequencing and transformed into BL21 (DE3). Expression and purification of mutants were carried out in the same way as for the wild type enzyme.

**Assay for Hydrolytic Activity of Endo-D and Mutants**—The hydrolytic activity of each enzyme was determined using the fucosylated and nonfucosylated compounds (1 and 2), respectively. The enzymatic reaction was performed at 30°C with 0.3 mM substrate in a phosphate buffer (100 mM, pH 7.5, 10 μl)
containing an appropriate amount of enzyme (5 ng for Endo-D, 3.6 ng for SpGH85, 100 ng for N322A, 20 ng for N322Q, 10 ng for Y360F, or 5 ng for H371W). Aliquots (1 μl each) were taken at intervals, and the enzymatic reaction was quenched by mixing each aliquot with 50 μl of 0.1% trifluoroacetic acid. The resulting mixture was analyzed by reverse-phase HPLC (see Methods in supplemental material) to quantify the amount of substrate hydrolysis.

Assay for Transglycosylation Activity of Endo-D and Its Mutants—The transglycosylation activity of the enzyme was assayed as follows. A mixture of Manα,GlcNAc-oxazoline (5 mM) and Fmoc-Asn(Fucα,GlcNAc)-OH (0.5 mM) or Fmoc-Asn(GlcNAc)-OH (0.5 mM) in a sodium phosphate buffer (50 mM, pH 7.5, 5 μl) containing 10% DMSO was incubated with spGH85 (0.19 μg), Endo-D (0.01 μg), or its mutant (0.01 μg), respectively at 30 °C. DMSO was added to enhance the solubility of the Fmoc-Asn(GlcNAc)-OH substrate in the aqueous buffer. Aliquots were taken at intervals, and the enzymatic reaction was analyzed by RP-HPLC as described above. The yield of the transglycosylation product was calculated by integration of the peak areas and normalized with the absorbance as follows: transglycosylation yield (%) = (product area/ (product area + residual acceptor area)) × 100.

Kinetic Studies on Transglycosylation by N322A and N322Q Mutants—To determine the constants (Km and kcat) for Manα,GlcNAc-oxazoline, Fmoc-Asn(Fucα,GlcNAc)-OH was used as an acceptor at a fixed concentration of 28.4 mM, and the concentration of Manα,GlcNAc-oxazoline was varied from 0.63 to 10 mM. The reaction was performed in phosphate buffer (100 mM, pH 7.5, 5 μl) containing 10% DMSO incubated at 30 °C for 10 min with 1 μg of N322A or 0.05 μg of N322Q. Each experimental point was quenched by adding 0.1% trifluoroacetic acid, the mixture was analyzed by RP-HPLC, and the transglycosylation product was quantified as described above. The parameters (Km and kcat) for Fmoc-Asn(Fucα,GlcNAc)-OH were determined with Manα,GlcNAc-oxazoline fixed at 2.5 mM, and Fmoc-Asn(Fucα,GlcNAc)-OH was varied at five concentrations between 1.77 and 28.4 mM. To determine the parameters for Fmoc-Asn(GlcNAc)-OH, Manα,GlcNAc-oxazoline was also fixed at 2.5 mM, and the concentration of Fmoc-Asn(GlcNAc)-OH was varied at five concentrations between 0.89 and 14.2 mM. The Km and Vmax values were obtained by fitting the experimental data into the Michaelis-Menten kinetics model using the GraphPad Prism software (GraphPad Software, Inc.).

Papain Digestion of Antibody Rituximab—The antibody was digested with protease papain according the reported method (34), with some modifications. Briefly, a solution of rituximab (20 mg) in a Tris-Cl buffer (20 mM, pH 6.5, 20 ml) containing L-cysteine (2 mM) was incubated with papain (200 μg) at 37 °C. The reaction was monitored by SDS-PAGE and LC-MS. When the production of an Fc fragment reached plateau (after 2 h), the reaction mixture was loaded 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 Fc fragments were then eluted 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) to give IgG–Fc (3.5 mg). As determined by LC-MS: calculated for nonglycosylated IgG–Fc dimer, Mz = 49,896 Da; found (m/z) (deconvoluted data), 52,763 (G0F homodimer), 52,925 (G0F/G1F heterodimer), 53,084 (G1F homodimer), and 53,400 (G2F homodimer).

Deglycosylation of Fc Fragments by Endo-S to Prepare Fuca1,6GlcNAc-Fc—A solution of the purified IgG–Fc (2 mg) in a Tris-Cl buffer (50 mM, pH 7.0, 0.4 ml) was incubated with Endo-S (20 μg) at 30 °C. After 30 min, the SDS-PAGE and LC-MS indicated the completion of the deglycosylation. The product was then purified through protein A affinity chromatography following the procedures described above to give the Fuca1,6GlcNAc-Fc (2 mg, quantitative yield). As determined by ESI-MS: calculated for Fuca1,6GlcNAc-Fc, Mz = 50,594; found (m/z) (deconvoluted data), 50,573.

Transglycosylation to Fuca1,6GlcNAc-Fc by Endo-D-N322Q—A solution of Fuca1,6GlcNAc-Fc (506 μg, 10 nmol) and Manα,GlcNAc-oxazoline (138 μg, 200 nmol) in a Tris buffer (50 mM, pH 6.8, 50 μl) was incubated with the N322Q mutant (10 μg) at 30 °C. Aliquots were taken at intervals and were analyzed by LC-MS. After 5 h, LC-MS indicated the completion reaction of Fuca1,6GlcNAc-Fc to give a new species corresponding to the transglycosylation product. The reaction mixture was injected into LC-MS, and the molecular weight of transglycosylation product was determined by ESI-MS: calculated for Manα,GlcNAc(α1,6Fuc)GlcNAc-Fc homodimer, Mz = 51,951; found (m/z) (deconvoluted data), 51,946.

RESULTS
Cloning and Expression of Endo-D and Selected Mutants—The full-length Endo-D is a large protein consisting of 1646 amino acid residues (Mz = 178,000), which is encoded by a gene of 4941 bp in length (7, 35). It has been previously reported that a truncated form (a.a. 135–1047) of the wild type Endo-D, in which 134 and 599 amino acids from the N and C terminus were removed, respectively, still retains enzymatic activity comparable with the full-length wild type enzyme (35). In the present study, we selected this truncated form of enzyme (hereafter called Endo-D) as the template for site-directed mutagenesis because of its much smaller size than the full-length wild type Endo-D. We also cloned an extensively truncated Endo-D form (amino acid sequence 159–807) for comparative studies. This further truncated form, the catalyzed domain of Endo-D termed spGH85, was recently expressed and used for x-ray crystallographic analysis and for transglycosylation study (16, 30).

Sequence alignment of Endo-D, Endo-A, and Endo-M led to the identification of several interesting residues that are likely to be directly involved in the substrate-assisted mechanism of catalysis by the GH85 enzymes (Fig. 1). These include Asn-171 in Endo-A and Asn-175 in Endo-M, which were previously identified as the key residue for orientating and promoting oxazoline formation in glycosidic bond hydrolysis (14, 15, 23, 24). The Glu-324 residue was equivalent to the Glu-173 of Endo-A and the Glu-177 in Endo-M,
which was identified as the general acid/base residue in the catalysis (14, 15, 23, 24). The recent x-ray structural study of spGH85 also confirmed the essential roles of Asn-322 and Glu-324 in the substrate-assisted mechanism of catalysis (the two residues were numbered as Asn-335 and Glu-337 in the x-ray crystal structure) (16). The Tyr-360 residue in Endo-D was aligned with Tyr-205 in Endo-A and Tyr-217 in Endo-M, mutation of which to a Phe residue was previously shown to significantly enhance the transglycosylation efficiency (23). The alignment of His-371 in Endo-D to the residues Trp-216 and Trp-228 in Endo-A and Endo-M, respectively, came as a surprise as Trp-216 in Endo-A was shown to be essential for transglycosylation but dispensable for hydrolytic activity (36). Accordingly, we generated five mutants (N322A, N322Q, E324Q, Y360F, and H371W) by site-directed mutagenesis on these four sites to probe the effects of these mutations on the transglycosylation activity of Endo-D.

The genes encoding Endo-D (a.a. 135–1047) and spGH85 (a.a. 159–807) were amplified from the genomic DNA of *S. pneumoniae* by PCR and cloned into the expression vector, pET28a. Endo-D mutants were created on the pET28a-EndoD template (encoding a.a. 135–1047) using the GENEART site-directed mutagenesis kit by designing appropriate pairs of primers. Endo-D, spGH85, and the mutants were then overproduced in the transformed *E. coli* strain BL21 (DE3) and purified to homogeneity on a nickel affinity column. The Endo-D (a.a. 135–1047) and its mutants appeared as a single band at $\sim 100$ kDa (supplemental Fig. 1, lanes 1–6), which was in good agreement with the expected molecular weight (calculated, $M_r = 102,000$). The extensively truncated Endo-D fragment, spGH85, appeared as a single band at $\sim 73$ kDa (supplemental Fig. 1, lane 7), which was consistent with the calculated molecular mass ($M_r = 74,500$).

**Hydrolytic Activity of Endo-D and Its Mutants**—Two synthetic substrates were used to assess the hydrolytic activity of the recombinant enzymes: the core-fucosylated N-pentasaccharide derivative (1) and the nonfucosylated derivative (2) (Fig. 2A). Upon hydrolysis, 1

![FIGURE 1. Alignment of amino acid sequence of catalytic core of Endo-D, Endo-A, and Endo-M. Residues Asn-322, Glu-324, Tyr-360, and His-371 of Endo-D are highlighted in dark gray.](image)

**FIGURE 2. Hydrolysis rate of Endo-D and its mutants using synthetic substrates.** A, reaction scheme. B, hydrolysis rates of fucosylated substrate Fmoc-Asn(Man,GlcNAc,Fuc)OH (1). C, hydrolysis rate of nonfucosylated substrate Fmoc-Asn(Man,GlcNAc)OH (2). The hydrolysis rates were determined using 2.8 mM of each substrate and quantified by RP-HPLC.
Tag was introduced to the Asn residue to facilitate HPLC monitoring of the enzymatic reactions. It was found that the spGH85, an extensively truncated form (a.a. 159–807), was much less active than Endo-D in hydrolyzing the core-fucosylated substrate (1) (Fig. 2B). The hydrolysis rate of Endo-D was about 4-fold lower than that of spGH85, indicating a loss of ~80% activity due to the further deletions of amino acid residues from the N and C terminus. This result is consistent with a previous study indicating that although the Endo-D (a.a. 135–1047) retained activity comparable with the full-length wild type enzyme, the further truncated Endo-D fragments (a.a. 1–966 and a.a. 201–1646) almost lost all the hydrolytic activity on the fucosylated N-pentasaccharide core (35). The N322A, N322Q, and E324Q mutants showed no or only residual hydrolytic activity, confirming the crucial role of these two residues for hydrolysis. In comparison with wild type Endo-D, the Y360F mutant demonstrated a decreased hydrolytic activity, whereas the H371W mutant showed a slightly enhanced hydrolytic activity on the core-fucosylated substrate (1) (Fig. 2B). When the nonfucosylated substrate (2) was used as the substrate, the difference in activity between Endo-D and spGH85 was much smaller than that for the fucosylated substrate (1), whereas the Y360F and H371W mutants showed about the same activity as the Endo-D itself (Fig. 2C). On the other hand, the mutants N322A and N322Q demonstrated only marginal activity on substrate (2), and the E324Q did not show any detectable activity on substrate (2). These data again confirm the critical roles of the two residues for catalyzing the hydrolysis.

Transglycosylation Activity of Endo-D, spGH85, and Selected Endo-D Mutants—To assess the transglycosylation activities of Endo-D and its mutants, we performed the transglycosylation reaction using Man3GlcNAc-oxazoline (5) as the donor substrate and Fmoc-Asn(Fucα1,6GlcNAc)-OH (3) and Fmoc-Asn(GlcNAc)-OH (4) as the acceptor substrates, respectively (Fig. 3A). The enzymatic reactions with Endo-D, spGH85, and the mutants were carried out under the same conditions (phosphate buffer, 50 mM, pH 7.5, donor/acceptor, 10:1). The transglycosylation reactions were monitored by HPLC analysis, and the yield was calculated on the basis of conversion of the acceptor substrate to the product (Fig. 3, B and C). The Endo-D and spGH85 showed only low transglycosylation efficiency for both the fucosylated and the nonfucosylated GlcNAc acceptors (3 and 4), and the transglycosylation product was gradually hydrolyzed (Fig. 3, B and C). In comparison, Endo-D and spGH85 showed a higher efficiency for transglycosylation on the GlcNAc acceptor (4) than the fucosylated GlcNAc acceptor (3). The transglycosylation of Endo-D on the fucosylated acceptor was marginal (less than 5% at the maximum under the assay conditions), which is consistent with our previous observations on wild type Endo-D (31). The extensively truncated enzyme, spGH85, gave a maximal yield of 20% when GlcNAc acceptor was used, which was similar to the previously reported results (30), whereas the less truncated enzyme (Endo-D) gave a maximal yield of 11%. In both cases, the product was hydrolyzed at the end. The Y360F mutant showed an enhanced transglycosylation activity and reduced product.

FIGURE 3. Transglycosylation activity of Endo-D and mutants. A, reaction scheme. B, Fmoc-Asn(Fucα1,6GlcNAc)-OH (3) as the acceptor. C, Fmoc-Asn(GlcNAc)-OH (4) as the acceptor. Square, N322Q; diamond, H371W; triangle, Y360F; star, N322A; cross, E324Q; open square, Endo-D; open diamond, spGH85. The transglycosylation reaction was carried out using 5 mM Man3GlcNAc-oxazoline as donor and 0.5 mM of each acceptor. The yields were calculated by the ratio of product/acceptor in RP-HPLC.
hydrolysis activity in comparison with Endo–D. The H371W mutant also demonstrated significantly enhanced transglycosylation efficiency, indicating that changing the His-371 to a Trp residue, as found at the equivalent position in Endo-A and Endo-M, did enhance the transglycosylation activity. Nevertheless, the Y360F and H371W mutants would still gradually hydrolyze the product given a prolonged time (data not shown). The E324Q mutant did not show any transglycosylation activity under the reaction condition, indicating the critical role of the Glu-324 residue for both hydrolysis and transglycosylation. The N322A mutant showed a moderate transglycosylation activity but, in contrast to Y360F and H371W, the N322A mutant proceeded with a steady increase of the transglycosylation yield without product hydrolysis even when the reaction was extended for 20 h (data not shown). The N322Q mutant demonstrated the highest transglycosylation activity, reaching 90% yield at 4 h for the fucosylated GlcNAc acceptor (Fig. 3B) and 1 h for the nonfucosylated GlcNAc acceptor (Fig. 3C). N322A and N322Q thus act as typical glycosynthases that take the activated sugar oxazoline for transglycosylation but lack the activity to hydrolyze the product. The N322A and N322Q mutants represent the first glycosynthases that can use an α-1,6-fucosylated GlcNAc moiety as acceptor for transglycosylation to form core-fucosylated N-glycopeptides. Interestingly, Endo-D and its mutants so far tested demonstrated much higher transglycosylation efficiency with the nonfucosylated acceptor (4) than the corresponding fucosylated acceptor (3). In particular, N322Q showed extraordinarily high transglycosylation efficiency.

Kinetic Studies on Transglycosylation by N322A and N322Q Mutants—The initial transglycosylation activity screening indicated that the N322A and N322Q mutants were two promising glycosynthases capable of catalyzing transglycosylation but having no or only residual product hydrolysis activity. In addition, the N322Q mutant showed apparently much higher transglycosylation initial rate than the N322A mutant. To understand the mechanism behind the observed kinetic difference of N322A and N322Q mutants, we determined the kinetic parameters of the transglycosylation catalyzed by the two mutants. The results were summarized in Tables 1 and 2. It was found that N322Q and N322A had a similar $K_m$ value (0.5–0.6 mM) for the donor substrate, Man$_3$GlcNAc-oxazoline, suggesting that both mutants have about the same affinity for the donor substrate. However, the $k_{cat}$ of N322Q was remarkably higher (over 48-fold) than that of N322A for the donor substrate (Table 1). Comparison of the kinetic data for the fucosylated and nonfucosylated GlcNAc acceptors (3 and 4) also revealed several interesting points (Table 2). The $K_m$ of N322Q for the fucosylated acceptor (3) was 24-fold higher than that for the nonfucosylated substrate (4), whereas its $k_{cat}$ for the fucosylated acceptor (3) was about 2-fold that of the nonfucosylated acceptor (4). These data suggest that N322Q mutant has a much lower affinity for the fucosylated GlcNAc acceptor. As a result, the transglycosylation efficiency of N322Q on the nonfucosylated substrate (4) was about 10-fold higher than that on the fucosylated substrate (3), as estimated by the $k_{cat}/K_m$ values (8.7 mM$^{-1}$s$^{-1}$ for 4 and 0.77 mM$^{-1}$s$^{-1}$ for 3, respectively). The enhanced catalytic efficiency of N322Q for the nonfucosylated substrate mainly comes from the dramatic enhanced affinity of N322Q for the nonfucosylated substrate. In contrast, the N322A had similar $k_{cat}$ and $K_m$ values for both fucosylated and nonfucosylated GlcNAc acceptors. The catalytic efficiency of N322A on the nonfucosylated substrate (4) was about 2-fold higher than that of the fucosylated substrate (3). By a cross comparison of the two mutants, the N322Q mutant was much more efficient than the N322A mutant for both types of substrates. The catalytic efficiency (as estimated by the $k_{cat}/K_m$ values) of N322Q is 27-fold higher than that of the N322A mutant for the fucosylated substrate (3), which was mainly contributed from the much higher turnover rate ($k_{cat}$) of the N322Q mutant. The catalytic efficiency of N322Q was about 90-fold higher than that of N322A mutant for the nonfucosylated substrate (4), which was contributed by both the higher turnover rate and the higher affinity of N322Q than that of the N322A mutant. Taken together, these data suggest that the N322Q mutant is a superior glycosynthase for the synthesis of both core-fucosylated and nonfucosylated glycopeptides or glycoproteins.

**Glycosylation Engineering of IgG1-Fc Using Endo-D Mutant—**The ability of N322Q mutant to transfer an N-glycan core to the fucosylated GlcNAc-Asn derivative prompted us to test whether the mutant was also efficient for glycosylation remodeling of IgG-Fc domain, as demonstrated in Fig. 4. For this purpose, we prepared human IgG-Fc fragment by papain digestion of rituximab, a commercial monoclonal antibody widely used for the treatment of lymphomas, following the previously reported procedure (34). The IgG-Fc fragment was purified by protein A affinity chromatography and was then deglycosylated.

### TABLE 1

| Mutant   | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|----------|-----------|-------|---------------|
| N322Q    | 10.17 ± 0.18 | 0.50 ± 0.07 | 20.4 |
| N322A    | 0.21 ± 0.01 | 0.67 ± 0.13 | 0.31 |

*a The $k_{cat}$ and $K_m$ values represent mean ± S.D. (n = 3).*

### TABLE 2

| Mutant   | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|----------|-----------|-------|---------------|
| N322Q    | 14.32 ± 0.98 | 18.51 ± 2.43 | 0.77 |
| N322A    | 0.24 ± 0.05 | 8.51 ± 4.16 | 2.86 × 10$^{-2}$ |

*a The $k_{cat}$ and $K_m$ values represent mean ± S.D. (n = 3).*
using Endo-S, an endoglycosidase from S. pyogenes that is specific for IgG deglycosylation (32). The resulting deglycosylated IgG-Fc ((Fucα1,6)GlcNAc-Fc) was a homodimer, in which the innermost disaccharide unit (Fucα1,6GlcNAc) remained at each of the Fc glycosylation sites. SDS-PAGE analysis of the reduced Fc fragments (Fc monomers) before and after deglycosylation indicated that the deglycosylated monomeric Fc (under reduced conditions) appeared as a single band that was about 1.4 kDa smaller than the original IgG-Fc monomer, suggesting the removal of a typical N-glycan from each of the Fc glycosylation sites (Fig. 5A, lane 1, natural complex type-Fc; lane 2, deglycosylated IgG-Fc). The transglycosylation of (Fucα1,6)GlcNAc-Fc with Manα1,3GlcNAc-oxazoline (5) as the donor substrate and N322Q mutant as the enzyme was monitored by SDS-PAGE (Fig. 5A, lanes 3–8). After 30 min, about half of the Fc acceptor was glycosylated as indicated by a new protein band, which appeared about 1 kDa larger than the precursor (Fig. 5A, lane 4). The reaction reached about 90% of conversion at 2 h (Fig. 5A, lane 6) and went to completion at 5 h (Fig. 5A, lane 8). It should be mentioned that a relatively low molar ratio of donor to acceptor (4:1, oxazoline to GlcNAc of Fc homodimer) could still lead to a 80% conversion at 5 h (data not shown), indicating the high catalytic efficiency of the Endo-D mutant. The glycosylation identity of the Fc fragments before and after remodeling was confirmed by mass spectrometric analysis. MALDI-TOF MS analysis of the Fc N-glycans released by N-glycosidase F revealed that the native N-glycans in the Fc dimer obtained from rituximab were three core-fucosylated biantennary complex type oligosaccharides carrying 0, 1, and 2 terminal galactose residues (termed as G0F, G1F, and G2F glycans) (data not shown). ESI-MS of the intact Fc dimer showed four major glycoforms: 52,763 (G0F homodimer), 52,925 (G0F/G1F heterodimer), 53,084 (G1F homodimer), and 53,404 (G2F homodimer) (Fig. 5B). Endo-S-catalyzed deglycosylation converted it into a single species, the ESI-MS data (m/z, 50,573), which matched with the Fc homodimer carrying two Fucα1,6GlcNAc disaccharides (calculated, Mr = 50,594) (Fig. 5C). The transglycosylation product appeared as a single peak in the ESI-MS (m/z, 51,946), which was in good agreement with the Fc homodimer carrying two core-fucosylated N-pentasaccharides (calculated, Mr = 51,951) (Fig. 5D). We also tested the activity of Endo-D mutants (N322A and N322Q) on full-size complex type N-glycan oxazolines, including (Galβ1,4)-GlcNAc(β1,2))2Manα1,3GlcNAc and (Siaα2,6Galβ1,4GlcNAc(β1,2))2Manα1,3GlcNAc oxazolines, but no transglycosylation product was detected. These results are consistent with the previously reported substrate specificity of Endo-D in hydrolysis, which shows that Endo-D cannot hydrolyze complex type N-glycan core unless the terminal sialic acid and that the intergalactose and GlcNAc residues are removed by α-sialidase, β-galactosidase, and β-N-acetylglucosaminidase sequentially (7, 35).

**DISCUSSION**

In this work, we have evaluated the hydrolysis and transglycosylation activity of recombinant Endo-D and its selected mutants. Our experimental data show that Endo-D can hydrolyze both fucosylated and nonfucosylated N-glycan cores, but the fucosylated N-glycan is a more favorable substrate than the nonfucosylated N-glycan for Endo-D-catalyzed hydrolysis (Fig. 2). The extensively truncated form of Endo-D (a.a. 159–807), which is the proposed catalytic domain (spGH85) used in a previous crystallographic study (16), was much less active than the less truncated Endo-D (a.a. 135–1047) (Fig. 3). Mutation at the Asn-322 and Glu-324 residues abolished the hydrolytic activity of the enzyme, confirming the essential roles of the two residues in Endo-D-catalyzed hydrolysis. These results are consistent with the recent x-ray crystal structural analysis (16) and the previously reported mutational studies (35).

For the transglycosylation, we have found that the Endo-D and related mutants clearly prefer the nonfucosylated GlcNAc to the fucosylated GlcNAc as the acceptor (Fig. 3). Previous studies showed that Endo-D and the recombinant catalytic domain (spGH85) gave a low overall transglycosylation yield, probably because of quick enzymatic hydrolysis of the sugar oxazoline and/or the transglycosylation product (30, 31). In the present study, we have generated several mutants, including Y360F, H371W, N322A, and N322Q, which show significantly enhanced transglycosylation efficiency. In particular, the N322A and N322Q mutants demonstrate remarkable transglycosylation activity with only marginal product hydrolysis activity, leading to an excellent yield of transglycosylation. The two mutants represent the first glycosynthases derived from Endo-D. Kinetic studies have revealed interesting features of the two glycosynthase mutants in terms of their substrate specificity and catalytic efficiency. Both the N332Q and the N322A mutants prefer the nonfucosylated GlcNAc (4) as the acceptor for transglycosylation. For the N322Q mutant, the catalytic efficiency (kcat/Km) on the nonfucosylated GlcNAc acceptor (4) is 10-fold higher than that of the fucosylated GlcNAc acceptor (3) (Table 2). This difference is mainly attributed to the much enhanced affinity of the mutant to the nonfucosylated acceptor (Kd of 0.73 mM for 4 versus Kd of 18.5 mM for 3). The decreased affinity of N322Q for the fucosylated GlcNAc acceptor is most likely due to the steric hindrance caused by the attachment of the α1,6-linked fucose to the GlcNAc acceptor. We have previously shown that Endo-A, Endo-M, and their corresponding mutants did not recognize core-fucosylated GlcNAc acceptor.

**FIGURE 4.** Glycosylation remodeling of IgG-Fc through transglycosylation with Endo-D mutants.
for transglycosylation (31). Taken together, these results suggest that the ability of Endo-D mutants to glycosylate fucosylated GlcNAc acceptor is most likely due to a more flexible space at the catalytic site in Endo-D to accommodate the fucose than in Endo-A or Endo-M, rather than due to favorable interactions between the fucose residue and the enzyme. We performed the alignment of the available crystal structures of Endo-D (16) and Endo-A (14, 15) and found that indeed Endo-D seemed to have a much more opened space for the acceptor recognition site than in Endo-A (data not shown). On the other hand, Endo-D did not recognize the complex type N-glycan oxazoline or Man9GlcNAc-oxazoline for transglycosylation, which is consistent with its substrate specificity in N-glycan hydrolysis.

Comparison of the N322Q and N322A mutants indicates that the N322Q mutant is much more efficient for catalyzing transglycosylation than the N322A mutant. This was reflected by the much enhanced turnover rate ($k_{cat}$) of the N322Q mutant over the N322A mutant for both sugar oxazoline and the acceptor substrates (Tables 1 and 2). In addition, in the case of the nonfucosylated acceptor (4), the N332Q mutant also shows much enhanced affinity for the substrate over the N322A mutant, as estimated by the $K_m$ value (Table 2). These data suggest that although replacement of the Asn-322 with both the Gln and the Ala residue disables its ability to promote oxazolinium ion intermediate formation for product hydrolysis, the Gln residue can better mimic the Asn-322 in promoting the later stage transglycosylation by facilitating the turnover and, in the case of the nonfucosylated GlcNAc acceptor, significantly enhancing the recognition of the acceptor substrate. The N322Q is particularly efficient for glycosylating the deglycosylated Fc domain having a Fuc$\alpha$1,6GlcNAc disaccharide moiety at each of the glycosylation sites. Because Endo-A and Endo-M are unable to recognize the Fucα1,6GlcNAc-Fc domain for transglycosylation (29), the N322Q mutant provides a valuable tool complementing well the existing endoglycosidase-based glycosynthases for remodeling IgG-Fc glycosylation, which is essential for the downstream effector functions of antibodies (37, 38).

In summary, the present study reveals interesting hydrolysis and transglycosylation properties of Endo-D and its selected mutants. Two novel glycosynthese mutants, N322Q and N322A, were generated that demonstrate remarkable transglycosylation efficiency with Man$_n$GlcNAc oxazoline but cannot...
act on full-length complex type N-glycan oxazoline, showing strict substrate specificity. The N322Q can efficiently glycosylate IgG-Fc domain carrying the Fuco(1,6)GlcNAc moiety. Studies on examining the Endo-D mutants for glycosylation remodeling of full-size IgG antibodies and on performing additional mutagenesis for broadening the substrate specificity of the mutants are in progress and will be reported in due course.

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