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# Generation of a Mutant *Mucor hiemalis* Endoglycosidase That Acts on Core-fucosylated *N*-Glycans*[S]

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**Endo-β-*N*-acetylglucosaminidase M (Endo-M), an endoglycosidase from the fungus *Mucor hiemalis*, is a useful tool for chemoenzymatic synthesis of glycoconjugates, including glycoprotein-based therapeutics having a precisely defined glycoform, by virtue of its transglycosylation activity. Although Endo-M has been known to act on various *N*-glycans, it does not act on core-fucosylated *N*-glycans, which exist widely in mammalian glycoproteins, thus limiting its application. Therefore, we performed site-directed mutagenesis on Endo-M to isolate mutant enzymes that are able to act on mammalian-type core-α1,6-fucosylated glycans. Among the Endo-M mutant enzymes generated, those in which the tryptophan at position 251 was substituted with alanine or asparagine showed altered substrate specificities. Such mutant enzymes exhibited increased hydrolysis of a synthetic α1,6-fucosylated trimannosyl core structure, whereas their activity on the afucosylated form decreased. In addition, among the Trp-251 mutants, the W251N mutant was most efficient in hydrolyzing the core-fucosylated substrate. W251N mutants could act on the immunoglobulin G-derived core-fucosylated glycopeptides and human lactoferrin glycoproteins. This mutant was also capable of transferring the sialyl glycan from an activated substrate intermediate (sialyl glycooxazoline) onto an α1,6-fucosyl-*N*-acetylglucosaminyl biotin. Furthermore, the W251N mutant gained a glycosynthase-like activity when a N175Q substitution was introduced and it caused accumulation of the transglycosylation products. These findings not only give insights into the substrate recognition mechanism of glycoside hydrolase family 85 enzymes but also widen their scope of application in preparing homogeneous glycoforms of core-fucosylated glycoproteins for the production of potent glycoprotein-based therapeutics.**

Endo-β-*N*-acetylglucosaminidases (ENGases,[3] EC 3.2.1.96) are enzymes that hydrolyze β-1,4 glycosidic linkages within the *N*,*N*′-diacetylchitobiose moiety in the *N*-glycan core structure and liberate a large part of the glycan, thus leaving the innermost *N*-acetylglucosamine residue (often attached to the core fucose) on the aglycon. These enzymes are distributed in a wide range of organisms, from bacteria living in various ecological niches to higher eukaryotes, including humans. In eukaryotes, the endoglycosidase activity of this enzyme is thought to be involved in the processing of free oligosaccharides in the cytosol (1–5), and in bacterial species, they may play roles in the acquisition of carbohydrates as energy sources from the environment (6, 7) or compromise the host defense system, contributing to the virulence of pathogenic bacteria (7–9).

There are two main classes of these endoglycosidases based on their amino acid sequences, within the carbohydrate-active enzymes (CAZy) database: glycoside hydrolase (GH) families GH18 and GH85. GH18 endoglycosidases, including the enzymes used frequently in glycoprotein analysis, such as Endo-H from *Streptomyces plicatus* (10), Endo-F1, -F2, and -F3 from *Elizabethkingia meningoseptica* (11), Endo-S from *Streptococcus pyogenes* (8), and Endo-T from *Tricoderma reesei* (12), normally show only hydrolytic activity, whereas GH85 enzymes, including Endo-M from *Mucor hiemalis*, Endo-A from *Arthrobacter protophormiae* (13), Endo-D from *Streptococcus pneumoniae* (14), Endo-Om from *Ogataea minuta* (15), and Endo-CC1 and -CC2 from *Coprinopsis cinerea* (16), show transglycosylation activity *in vitro* in the presence of suitable acceptor substrates as well as hydrolytic activity.

Based on mutation analyses of GH85 enzymes (17–19) and x-ray crystallographic studies of Endo-D (20) and Endo-A (21, 22), it has been postulated that the reaction mechanism of GH85 involves substrate-assisted catalysis. The reaction comprises two steps with the retention of anomeric configuration. In the first step, an enzymatic carboxylic acid (Glu-177 in Endo-M; Fig. 1*D*) acts as a general acid, with the concomitant nucleophilic attack by the carbonyl oxygen of the 2-acetamide group in *N*-acetylglucosamine (substrate), to form a sugar oxazoline intermediate. In the second step, the resulting intermediate is broken down by a process that is almost the reverse of

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[S] This article contains supplemental data, Tables S1 and S2, Scheme S1, and Fig. S1.

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[3] The abbreviations used are: ENGase, endo-β-*N*-acetylglucosaminidase; GH, glycoside hydrolase; Fuc, L-fucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; PA-, pyridylaminated; SG-oxazoline, sialylglyco-oxazoline; PNGase F, peptide:*N*-glycanase F; hLF, human lactoferrin.

the first step, accompanied either by hydrolysis (in the presence of water) or transglycosylation (in the presence of an appropriate acceptor molecule instead of water) (23). Abbott *et al.* (20) proposed a catalytic mechanism involving an unusual proton shuttle in which the imidic acid tautomer of another catalytically important residue, asparagine (Asn-175 in Endo-M), may be involved in the orientation of the 2-acetamido group of the substrate, and the Asn residue also acts as a general base to facilitate the formation of an oxazoline intermediate.

Endo-M serves as a particularly useful GH85 enzyme because it exhibits significant transglycosylation activity toward complex-type oligosaccharides, including sialo-oligosaccharides (24–29). Glycoforms of glycoproteins play important roles in regulating their biological activities. Especially, sialosides generally affect the half-lives of glycoproteins in the body fluid, and they often modulate immune responses (30–32). Thus, regulation of glycoforms in glycoprotein-based therapeutics (biomedicines), including antibodies and cytokines, have drawn attention in the pharmaceutical industry. This enzyme has been explored extensively to expand its utility, by protein engineering using site-directed mutagenesis, resulting in the isolation of efficient glycosynthase-like mutant enzymes, such as N175Q (17, 33, 34). These mutant enzymes exhibit both effective synthetic rates using sugar oxazoline as the glycosyl donor substrate and lack of hydrolytic activity, resulting in minimum degradation of the reaction products (33, 35, 36). Nonetheless, Endo-M and its mutants show limited activity toward core-fucosylated glycans.

Core fucosylation is a modification often found in natural and recombinant glycoproteins that affects *N*-glycan conformation and regulates their biological activity (37). Notably, IgG1 with the fucose-deficient *N*-glycan of Asn-297 in its Fc region shows increased binding affinity for the Fc$\gamma$ receptor III on the effector cells, resulting in highly enhanced antibody-dependent cellular cytotoxicity (38, 39). Endoglycosidase-catalyzed modification of core-fucosylated glycans has been demonstrated using glycosynthase mutants of Endo-D (40) and some GH18 enzymes. Transglycosylation onto $\alpha$1,6-fucosyl GlcNAc moieties by endoglycosidase was first reported in Endo-F2 and Endo-F3 (41). Recently, Endo-F3 was converted to a glycosynthase, and the mutant enzyme was capable of transferring bi- and triantennary complex type *N*-glycans using sugar oxazoline donor substrates to synthesize core-fucosylated complex glycopeptides (42). Endo-S and its glycosynthase, which specifically and efficiently acts on the IgG-Fc domain of *N*-glycans, have been used for chemoenzymatic synthesis of IgGs with structurally defined glycoforms for functional studies (43–45). More recently, Endo-S2 was shown to have a more flexible substrate specificity and high efficiency in transferring complex, hybrid, and high mannose-type *N*-glycans onto core-fucosylated or non-fucosylated IgG molecules (46).

In this study, we generated Endo-M mutant enzymes that are able to act on core-fucosylated substrates. These mutant enzymes, including W251N, exhibited an altered substrate preference for core-fucosylated glycan substrates, demonstrating their potential to act on more diverse glycoproteins. To the best of our knowledge, this is the first report that the substrate specificity of an endoglycosidase was altered by site-directed mutagenesis. These findings may provide new insights into the mechanism of the interaction between enzymes and glycan substrates, giving a basis for further endoglycosidase improvement by protein engineering.

## Results

*Structural Comparison of GH85 Enzymes*—To determine the amino acid residues critical for substrate preference of Endo-M with respect to $\alpha$1,6-linked core-fucosylated glycans, we first carried out a comparison of the GH85 enzymes. In light of the differences in substrate specificities and the availability of tertiary structures, we compared Endo-A (22) (Protein Data Bank entry 3FHQ) and Endo-D (20)(Protein Data Bank entry 2W92) (Fig. 1, *A*–C). Because the structural data for Endo-M are not available, we assumed Endo-A to be "virtual Endo-M," considering that neither of them cleave core-fucosylated glycans. On the other hand, Endo-D has been reported to act on core-fucosylated glycans when they are trimmed to the trimannosylated core structure ($Man_3GlcNAc_2Fuc_1$).

Comparison of the surface structures of the catalytic sites of Endo-A and Endo-D revealed differences in the distribution of amino acid residues. In Endo-A, a couple of tryptophan residues (Trp-216 and Trp-244) are distributed around a presumable subsite corresponding to the innermost GlcNAc residue of the *N*-glycan substrate, whereas in Endo-D, an asparagine (Asn-413) and a histidine (His-384) residue are present instead. Interestingly, these tryptophan residues of Endo-A are conserved in Endo-M (Trp-228 and Trp-251) (Fig. 1*D*). We also focused on a proline residue at position 128 and a tyrosine residue at position 131 of Endo-A, because they stretch over the glycan substrate bound to the catalytic site. These residues might determine substrate specificity; however, Pro-128 and Tyr-131 are not conserved in the amino acid sequence of Endo-M (Gly-125 and Gln-128) or in that of Endo-D (Trp-292 and Ser-295) (Fig. 1*D*). To examine the roles of these residues in determining the substrate preference of Endo-M, six Endo-M mutant enzymes (G125W, Q128A, Q128S, W228H, W251A, and W251N) were generated in an *Escherichia coli* expression system and used to investigate their substrate preferences.

*Evaluation of Hydrolytic Activities of Endo-M Mutants on Biotinylated Substrates*—The six mutants were assayed for hydrolytic activity using biotinylated trimannosyl core structure, with and without an $\alpha$1,6-linked core fucose residue ($Man_3GlcNAc_2Fuc_1$-biotin and $Man_3GlcNAc_2$-biotin, respectively) as the substrate (Fig. 2*A*). Thin layer chromatography (TLC) analysis demonstrated that the wild-type enzyme and all of the mutant enzymes (1 $\mu$g) could hydrolyze 10 $\mu$g of $Man_3GlcNAc_2$-biotin within 20 min (Fig. 2*B*). However, they could hardly hydrolyze $Man_3GlcNAc_2Fuc_1$-biotin, except the two Trp-251 mutants, W251A and W251N, which could hydrolyze most of the core-fucosylated substrate. The products were confirmed by MALDI-TOF/MS; the observed masses for $Man_3GlcNAc_1$ and Fuc$\alpha$1–6GlcNAc-biotin are *m*/*z* 730.6 $[M + Na]^+$ (calculated; *m*/*z* 730.2) and *m*/*z* 673.6 $[M + Na]^+$ (calculated; *m*/*z* 673.3), respectively (Fig. 2*C*). The reaction products were then quantified by HPLC analysis, and the specific activities of the mutants with each substrate were obtained. As

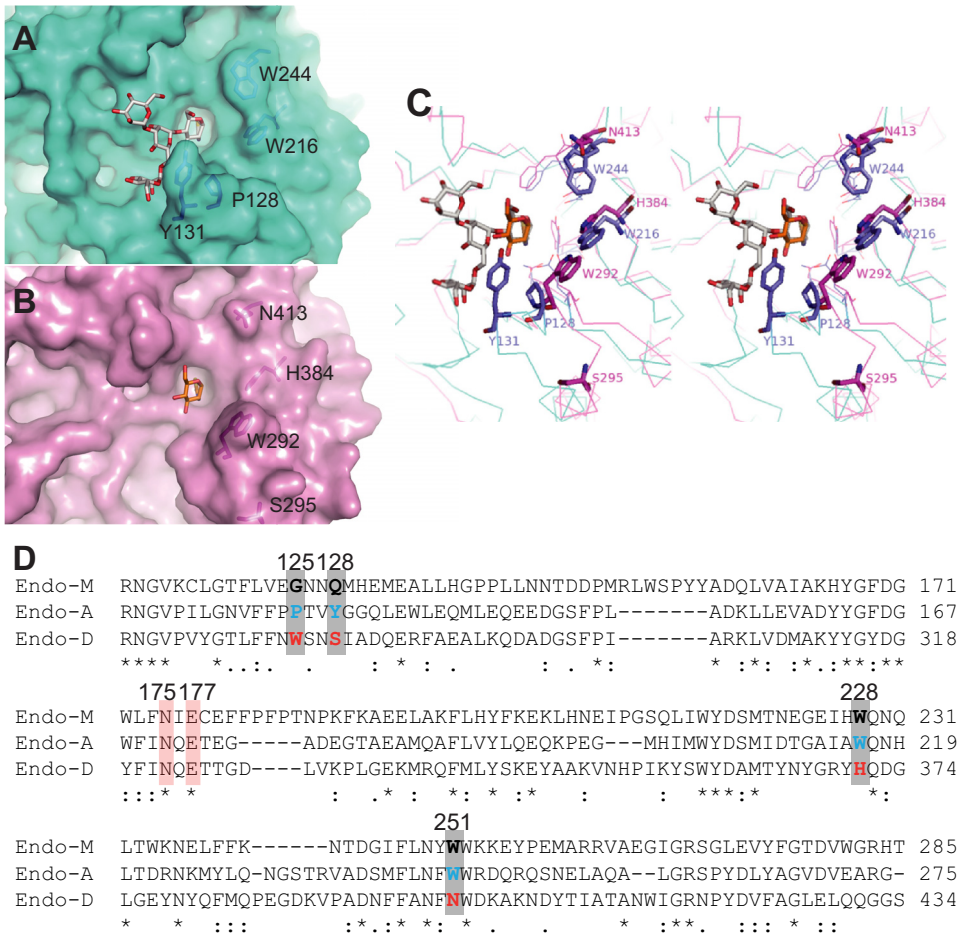


FIGURE 1. **Comparison of the protein structures of GH85 enzymes.** *A*, the protein surface structure around the catalytic core of Endo-A complexed with a trimannosyl thiazoline (Protein Data Bank accession number 3FHQ). *B*, the protein surface structure around the catalytic core of Endo-D complexed with a thiazoline (Protein Data Bank accession number 2W92). *C*, superimposition of the Endo-A and Endo-D structures (*stereo view*). The four amino acids of interest are indicated. *D*, alignment of the amino acid sequences around the proposed catalytic core of Endo-M, Endo-A, and Endo-D. Residues Gly-125, Gln-128, Trp-228, and Trp-251 of Endo-M are highlighted in *light gray*. Residues Asn-175 and Glu-177, the putative catalytic residues, are highlighted in *pink*.

shown in Table 1, all of the mutants exhibited lower specific hydrolytic activity with $Man_3GlcNAc_2$-biotin than the wild-type enzyme (6.49 $\mu$mol/min/mg). In particular, G125W and the two Trp-251 mutants showed an about 40-fold reduction. In contrast, the wild-type enzyme exhibited marginal specific hydrolytic activity with $Man_3GlcNAc_2Fuc_1$-biotin (<0.2% of the specific activity observed with the afucosylated substrate). G125W, Q128A, Q128S, and W228H showed undetectable or marginal activities, similar to the wild-type enzyme. However, consistent with the result of TLC analysis, W251A and W251N showed greater substrate preference for core-fucosylated trimannosyl glycans (0.13 and 1.3 $\mu$mol/min/mg, respectively) than the wild-type enzyme. W251N exhibited a 350-fold increase in specific hydrolytic activity with the core-fucosylated substrate, compared with the wild-type enzyme, indicating that Trp-251 of Endo-M is important for determining specificity for the glycan core structure. Because Trp-251 mutants showed increased activity with $Man_3GlcNAc_2Fuc_1$-biotin, we next examined the effect of replacement with an amino acid other than alanine and asparagine. All Endo-M Trp-251 variants, except W251C (the expression of which impaired *E. coli* growth), were generated, and their relative hydrolysis ratios for $Man_3GlcNAc_2$-biotin and $Man_3GlcNAc_2Fuc_1$-biotin were compared (Fig. 3). We found that W251N showed the highest activity with the core-fucosylated substrate among Trp-251 variants. Interestingly, the wild-type and W251N mutant enzymes showed a sharp contrast in their substrate preference (*i.e.* they prefer either afucosylated or core-fucosylated glycans). The W251F variant showed substrate preference rather like the wild-type enzyme, implying the involvement of aromatic side chains in substrate recognition; however, W251Y showed reduced activity with the afucosylated substrate. Furthermore, W251H retained ~20% activity with the afucosylated substrate and acquired hydrolytic activity with the core-fucosylated substrate.

*Alteration of Substrate Specificity of the W251N Mutant*—To investigate the possibility of its enzymatic application, the Endo-M W251N variant was further characterized. The optimal pH for hydrolysis (pH 6.0) was similar for the wild-type enzyme with $Man_3GlcNAc_2$-biotin and W251N with $Man_3GlcNAc_2Fuc_1$-biotin. The kinetic parameters of the wild-type enzyme and W251N variant with both substrates are shown in Table 2. There is little difference in the $K_m$ values for $Man_3GlcNAc_2$-biotin between the two enzymes. However, the $K_m$ value of W251N for the core-fucosylated substrate is >90-fold higher than that for the afucosylated one. The $k_{cat}$ values of

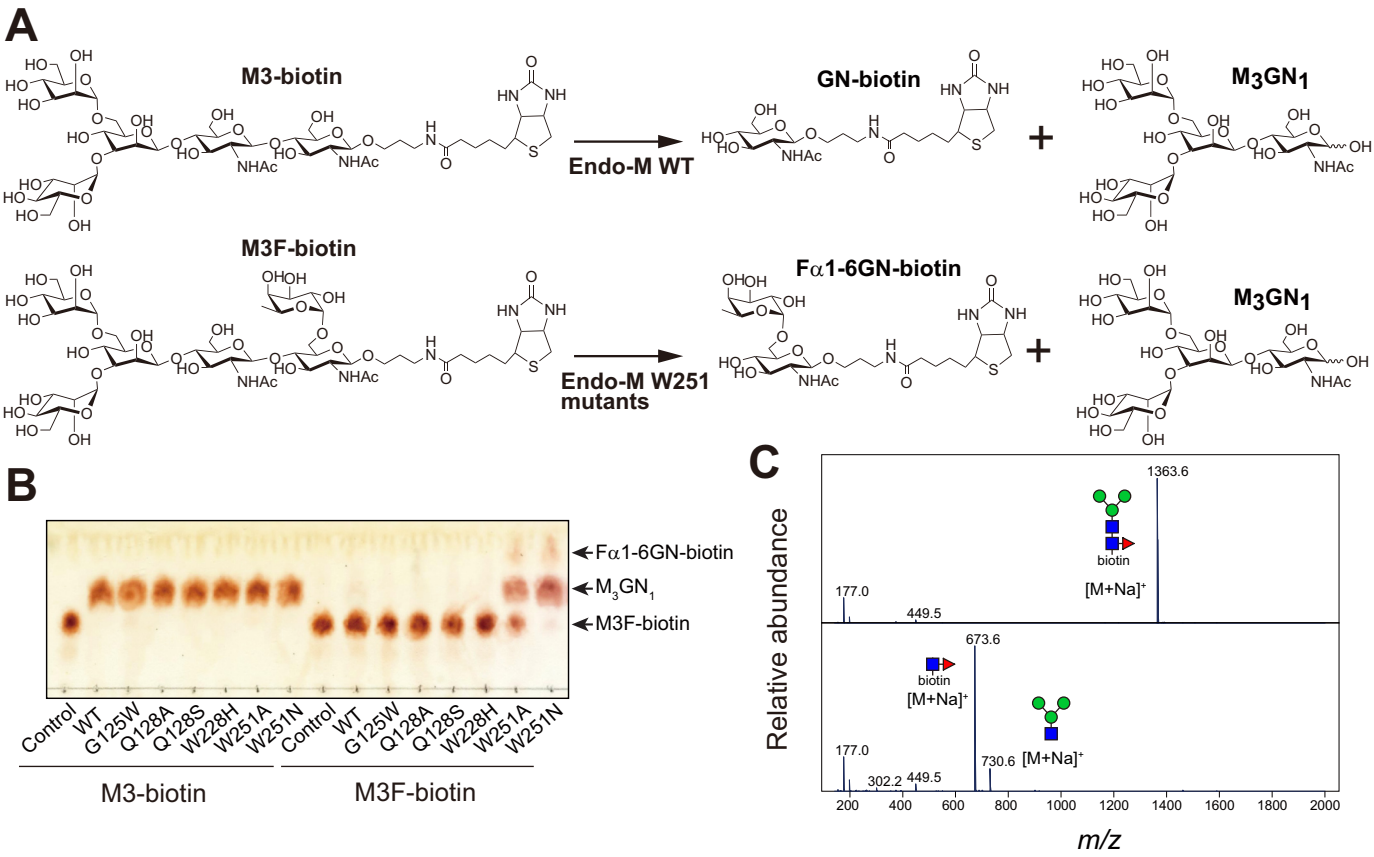


FIGURE 2. **Evaluation of the hydrolytic activity of Endo-M mutants using core fucosylated synthetic substrate.** *A*, schematic of the reaction of trimannosyl biotinylated synthetic substrates with and without core fucose, catalyzed by Endo-M. *M*, D-mannose; *GN*, D-GlcNAc; *F*, L-fucose. *B*, TLC analysis of the hydrolysis of each substrate by Endo-M variants. *C*, MALDI-TOF/MS analysis of the reaction mixtures of the W251N mutant enzyme with a core fucosylated synthetic substrate, $Man_3GlcNAc_2Fuc_1$-biotin. *Top*, before adding the enzyme; *bottom*, after incubation with the enzyme. Schematics of the estimated structures represent the following: D-GlcNAc (*blue square*), D-mannose (*green circle*), and L-fucose (*red triangle*).

**TABLE 1**
**Comparison of the hydrolysis activity of wild-type and mutant enzymes**

| Endo-M variants | $Man_3GlcNAc_2$-biotin | | $Man_3GlcNAc_2Fuc_1$-biotin | |
|---|---|---|---|---|
| | Specific hydrolysis activity[a] | Relative activity[b] | Specific hydrolysis activity[a] | Relative activity[b] |
| | *μmol min*$^{-1}$ *mg*$^{-1}$ | % | *μmol min*$^{-1}$ *mg*$^{-1}$ | % |
| Wild type | 6.49 | 100 | <0.01 | <0.2 |
| G125W | 0.15 | 2.4 | ND[c] | |
| Q128A | 0.82 | 13 | <0.01 | <0.2 |
| Q128S | 1.3 | 20 | <0.01 | <0.2 |
| W228H | 2.9 | 45 | <0.01 | <0.2 |
| W251A | 0.18 | 2.7 | 0.13 | 2.0 |
| W251N | 0.16 | 2.5 | 1.3 | 20 |

[a] The hydrolysis activity was determined by a single assay using 2 μg/ml enzyme with 3 mM $Man_3GlcNAc_2$-biotin or 20 μg/ml enzyme with 3 mM core fucosylated $Man_3GlcNAc_2$-biotin.
[b] The relative activity of each enzyme was calculated with respect to the activity of wild-type enzyme with $Man_3GlcNAc_2$-biotin as a substrate, which was set to 100.
[c] Not detected.

the wild-type and W251N enzymes for their respective preferred substrate are not very different.

Next, to determine whether the W251N substitution affects the specificity for non-reducing terminal structures of *N*-glycan substrates, we examined the relative activities with various pyridylaminated (PA-) glycan substrates (Table 3). The wild-type enzyme acted well on the afucosylated substrates, but not on the core-fucosylated glycans; its specific activities with the substrates were in the following decreasing order: Man3, Man5, asialo-, sialo-, and agalacto-biantennary complex-type glycans. However, the W251N variant, which could act on afucosylated PA-Man3 substrates unlike on afucosylated biotinylated substrates, showed ~30% of the wild-type enzyme's activity with afucosylated PA-Man3, thus showing a pattern similar to that of the wild-type enzyme; the order of preference for core-fucosylated substrates was similar between the wild-type enzyme and the W251N variant (M3F > asialo- > agalacto-). The difference between the enzymatic activities of W251N with biotinylated, afucosylated glycans and pyridylaminated ones is probably due to the open ring of the GlcNAc residue at the reducing termini. Taken together, the substrate specificity shifted from afucosylated glycans to core-fucosylated glycans, due to a single amino acid substitution, W251N, with minimum impact on the recognition of the structures at the non-reducing end.

*Hydrolytic Activity of W251N Variant on Glycopeptides and Glycoproteins*—We next examined whether the W251N mutant was able to release *N*-glycans having a core fucose from glycopeptide and glycoprotein substrates. Rituximab is an anti-CD20 IgG1 mAb, which is produced by a Chinese hamster ovary cell line. It is known that 95% of the *N*-glycans attached to Asn-297 of the Fc region of rituximab are core-fucosylated (47); we confirmed this by MS analysis of PNGase F-released *N*-glycans (Fig. 4*A* and supplemental Table S1). We prepared a mixture of *N*-glycopeptides/peptides from rituximab by tryptic digestion, for assaying the activity of the W251N mutant enzyme. The rituximab peptide mixture was incubated with Endo-M wild-type enzyme, the W251N variant, and the IgG-specific endoglycosidase Endo-S (8). The released glycans were collected, derivatized by permethylation, and analyzed by MALDI-TOF/MS (Fig. 4, *B–D*). In the reaction with the wild-type enzyme (Fig. 4*B*), the released *N*-glycans could be scarcely detected, due to the resistance of core fucosylated glycans to Endo-M wild-type enzyme, whereas they could be detected in the reaction with the W251N variant (Fig. 4*C*). As indicated in the figures, the MALDI-TOF/TOF analysis revealed that the deduced structures correspond to endoglycosidase-released structures with a single GlcNAc residue at the reducing ends of *N*-glycan. The glycan signal pattern was almost identical to that from the reaction mixture with Endo-S (Fig. 4*D*), indicating that W251N can act on most of the glycovariants of the IgG glycopeptide.

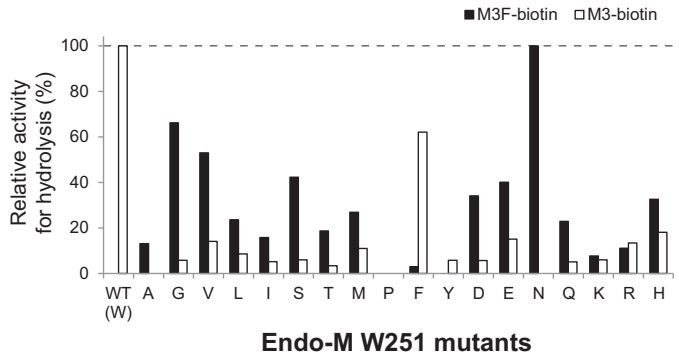


FIGURE 3. **Relative hydrolytic activities of Endo-M Trp-251 mutants on synthetic substrates.** Endo-M Trp-251 mutants were obtained by saturation mutagenesis, except the W251C mutant. The relative hydrolysis rates for both synthetic substrates ($Man_3GlcNAc_2$-biotin; *open bars*; $Man_3GlcNAc_2Fuc_1$-biotin; *filled bars*) were measured, and the highest values were taken as 100%. Data were obtained using a single assay.

Human lactoferrin (hLF) is a glycoprotein that has mostly core-fucosylated glycans at the two *N*-glycosylation sites (48, 49); this was confirmed by MS analysis (Fig. 5*A* and supplemental Table S2). We used hLF to characterize further the hydrolytic activity of W251N. Human LF was treated with Endo-S, wild-type Endo-M, and Endo-M W251N variant and then separated by SDS-PAGE and stained by Coomassie Brilliant Blue (Fig. 5*B*). In the untreated control, a major part of the hLF was observed slightly above an 80-kDa protein marker. Endo-S treatment removed only a single sugar chain, as revealed by the emergence of a lower band. This result was unchanged by treatment with increasing amounts of Endo-S, up to 1,000 units (~1.5 $\mu$g) (data not shown). Treatment with wild-type Endo-M (2 $\mu$g) showed a pattern similar to that of Endo-S treatment. On the other hand, upon treatment with Endo-M W251N variant, a new band emerged further below, indicating the presence of a completely deglycosylated form of hLF. Subsequent MALDI-TOF/MS analyses of permethylated glycans that were released by treatment with wild-type Endo-M and W251N confirmed that both enzymes could release various *N*-glycans, including Man5 and complex biantennary structures, from hLF (Fig. 5*C*). Note that the signal intensities of glycans from W251N enzyme treatment were greater than that of glycans from the wild-type enzyme treatment, probably due to the increased availability of core-fucosylated *N*-glycans; this is consistent with the result of the SDS-PAGE analysis.

**TABLE 2**

**Kinetic parameters of the hydrolysis of biotinylated substrates catalyzed by wild-type and W251N Endo-M**

| Endo-M | $Man_3GlcNAc_2$-biotin | | | $Man_3GlcNAc_2Fuc_1$-biotin | | |
|---|---|---|---|---|---|---|
| | $k_{cat}$ | $K_m$ | $k_{cat/K_m}$ | $k_{cat}$ | $K_m$ | $k_{cat/K_m}$ |
| | $s^{-1}$ | mM | $mM^{-1}\ s^{-1}$ | $s^{-1}$ | mM | $mM^{-1}\ s^{-1}$ |
| WT | 17.5 ± 2.5[a] | 0.39 ± 0.11 | 46 | ND[b] | ND | ND |
| W251N | 0.46 ± 0.04 | 0.21 ± 0.05 | 2.2 | 27.7 ± 0.40 | 19.6 ± 0.53 | 1.4 |

[a] The values are shown with S.E. ($n = 3$).
[b] ND, not determined.

**TABLE 3**

**Relative hydrolytic activity of wild-type Endo-M and W251N mutant on PA-oligosaccharides**

Various PA-oligosaccharides were used as the substrate for these assays.

| PA-oligosaccharide | Relative activity[a] | |
|---|---|---|
| | Wild type | W251N |
| | % | |
| PA-trimannosyl core (M3) | 100 | 100 |
| PA-oligomannose (M5) | 33 | 40 |
| PA-agalactosylbiantennary | 10 | 7.9 |
| PA-asialobiantennary | 21 | 19 |
| PA-sialobiantennary | 17 | 24 |
| PA-fucosyl trimannosyl core (M3F) | <0.1 | 18 |
| PA-fucosyl agalactosylbiantennary | <0.1 | 8.8 |
| PA-fucosyl asialobiantennary | <0.1 | 9.2 |

[a] The relative activity of each enzyme for a given PA-oligosaccharide was determined using a single assay and calculated with respect to the activity of M3, which was set at 100.

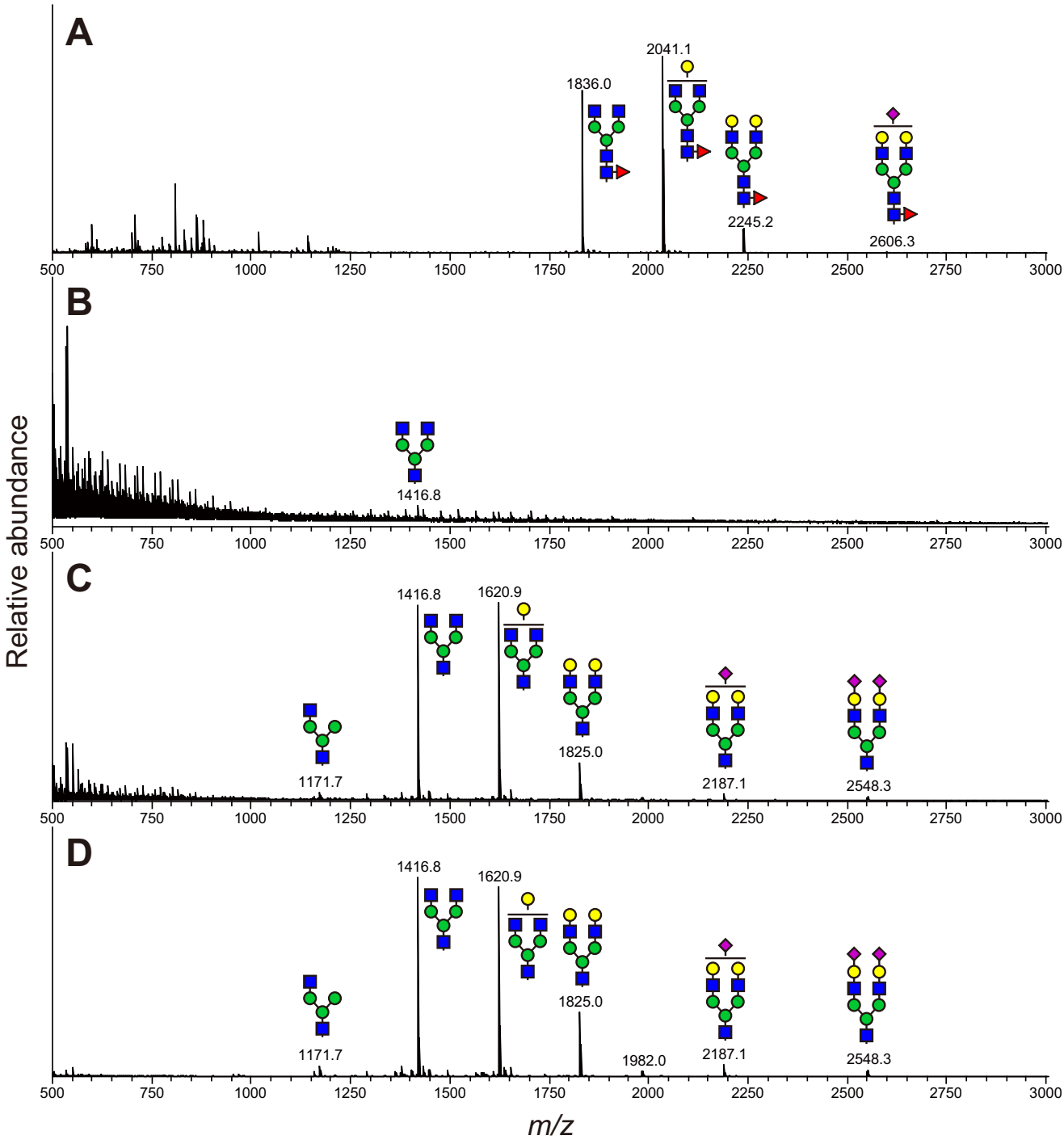


FIGURE 4. **MALDI-TOF/MS analyses of the glycans from rituximab glycopeptides, released by treatment with various enzymes.** Glycopeptides of rituximab were prepared and treated with PNGase F (*A*), wild-type Endo-M (*B*), W251N variant (*C*), and Endo-S (positive control) (*D*). The released oligosaccharides were collected, permethylated, and analyzed by MALDI-TOF/MS, as described under "Experimental Procedures." Schematics of the estimated structures represent the following: Neu5Ac (*purple diamond*), D-galactose (*yellow circle*), D-GlcNAc (*blue square*), D-mannose (*green circle*), and L-fucose (*red triangle*).

*Evaluation of the Transglycosylation Activity of W251N Variant and the Generation of Glycosynthase-like Enzyme N175Q/W251N*—One of the advantages of using Endo-M is that its transglycosylation activity enables the synthesis of novel sialoglycoconjugates. Hence, we next examined the ability of the W251N mutant enzyme to facilitate transglycosylation. Incubation of W251N with Fuc$\alpha$1–6GlcNAc-biotin as the acceptor and sialylglyco-oxazoline (SG-oxazoline) as the donor resulted in the detection of a new product peak in HPLC analysis. Fig. 6*A* shows a representative HPLC profile of the transglycosylation reaction products produced by the N175Q/W251N double mutant, which will be described below. The molecular mass of the product was *m/z* 2719 $[M + 3Na]^+$, according to MALDI-TOF/MS analysis. Moreover, in the MALDI-TOF/TOF-MS analysis of the product peak, the signature ions for sialylated glycans were detected, indicating that the product

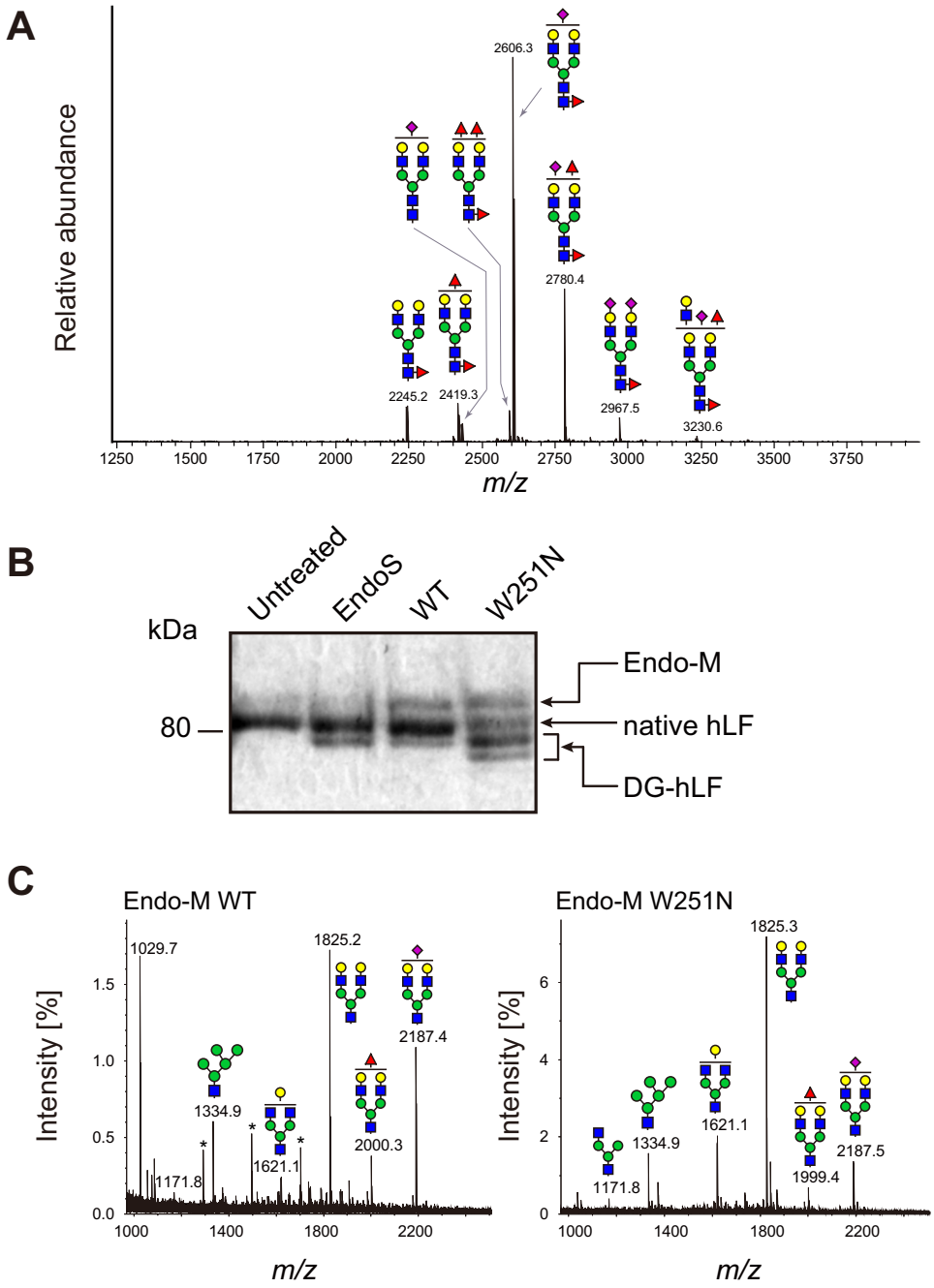


FIGURE 5. **Hydrolysis of the *N*-glycans of human lactoferrin by the W251N variant.** *A*, MALDI-TOF/MS spectrum of permethylated *N*-glycans released from the tryptic glycopeptides of human lactoferrin by PNGase F. *B*, hLF (10 $\mu$g) was treated with either Endo-S (100 units), wild-type Endo-M (2 $\mu$g), or Endo-M W251N mutant (2 $\mu$g), and then the aliquots were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The singly glycosylated hLF (*top* deglycosylated hLF (*DG-hLF*) band) was observed upon treatment with Endo-S and wild-type Endo-M, as indicated. However, the non-glycosylated hLF (*bottom* deglycosylated hLF band) as well as singly glycosylated hLF were detected upon treatment with the Endo-M W251N variant. *C*, MALDI-TOF/MS spectra of permethylated glycans released from hLF samples by wild-type Endo-M (*left*) and the W251N variant (*right*). The estimated glycan structures based on the *m/z* values of precursor ion mass and MS/MS fragment (data not shown) are depicted in the figures. Schematics of the estimated structures represent the following: Neu5Ac (*purple diamond*), D-galactose (*yellow circle*), D-GlcNAc (*blue square*), D-mannose (*green circle*), and L-fucose (*red triangle*). *Asterisks* indicate the hexose polymer peaks.

was a newly generated sialylglycoconjugate ($Neu5Ac_2$-$Gal_2GlcNAc_2Man_3GlcNAc_2Fuc_1$-biotin) (Fig. 6*B*). The product was hydrolyzed by Endo-F3, which can hydrolyze core-fucosylated biantennary complex-type *N*-glycans, but not by wild-type Endo-M, thus confirming that an *N*,*N*′-chitobiose core was formed in the transglycosylation reaction by W251N (data not shown). The yield of the transglycosylation product with 5 mM Fuc$\alpha$1–6GlcNAc-biotin and 5 mM SG-oxazoline (1:1 ratio) reached up to about 5% after a 2-h incubation; however, this was followed by a decline after a prolonged reaction time, presumably due to the degradation of the transglycosylation product by the inherent hydrolytic activity of the W251N enzyme (Fig. 6*C*). On the other hand, N175Q, an Endo-M glycosynthase-like mutant, scarcely synthesized the transglycosyl-

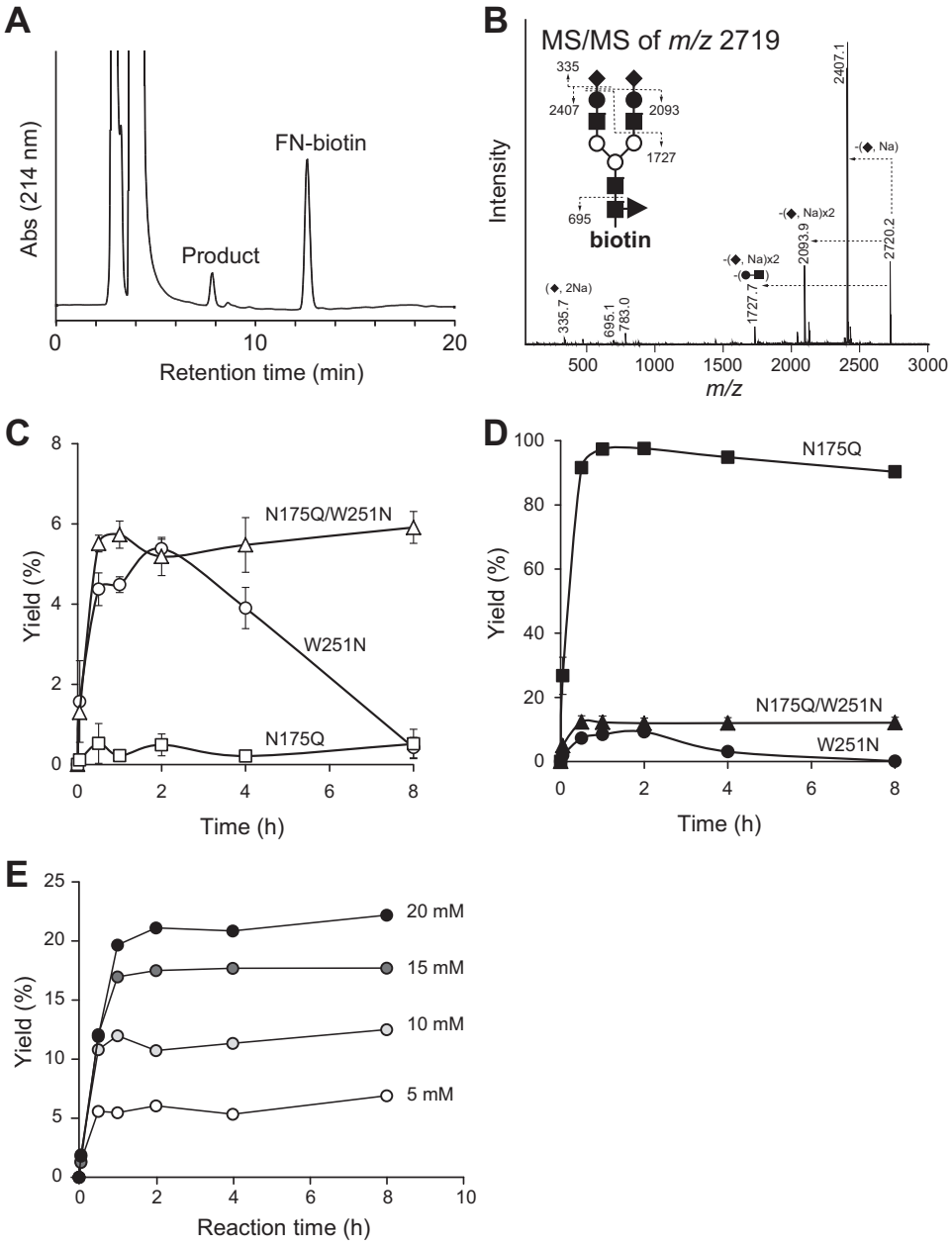


FIGURE 6. **Transglycosylation by the W251N mutant enzyme.** *A*, a reversed-phase HPLC profile of the transglycosylation reaction of N175Q/W251N mutant enzyme with Fuc$\alpha$1–6GlcNAc-biotin as the acceptor substrate and SG-oxazoline as the donor substrate after a 1-h incubation. *B*, MALDI-TOF/TOF-MS spectra of the transglycosylation products. A precursor MS peak at *m/z* 2719, $Neu5Ac_2Hex_5HexNAc_4dHex_1$-biotin, $[M + 3Na]^+$, was subjected to MALDI-TOF/TOF-MS analysis. The fragment ion peaks in the MS/MS spectrum of *m/z* 2719 correspond to the signature ions for sialylated glycans: loss of a terminal Neu5Ac plus a sodium ion (*m/z* 2407); loss of two pairs of terminal Neu5Ac plus a sodium ion (*m/z* 2093); loss of a Neu5Ac plus a sodium ion and a Neu5Ac-Hex-HexNAc moiety plus a sodium ion (*m/z* 1727); a sodium adduct of dHex-HexNAc-biotin (*m/z* 695); and a sodium adduct of Neu5Ac (*m/z* 335). Schematics of the structures represent the following: Neu5Ac (*diamond*), galactose (*filled circle*), GlcNAc (*filled square*), mannose (*open circle*), L-fucose (*triangle*). *C* and *D*, yields of the transglycosylation product in the reaction of Endo-M mutants with either Fuc$\alpha$1–6GlcNAc-biotin (*C*) or GlcNAc-biotin (*D*) as the acceptor substrate and SG-oxazoline as the donor substrate. Data represent the average of three independent reactions with mean ± S.D. (*error bars*). *E*, transglycosylation product yields from the reaction of the Endo-M N175Q/W251N double mutant with increased amounts of SG-oxazoline donor substrate in optimized reaction conditions.

ation product with the fucosylated acceptor (Fig. 6*C*). In contrast, with the GlcNAc-biotin as the acceptor substrate, N175Q generated >95% yield in a 1-h reaction with a marginal reduction of the product over the incubation time (Fig. 6*D*), suggesting that Trp-251 is critical for the recognition of Fuc$\alpha$1–6GlcNAc-biotin as an acceptor. Moreover, the relatively low yield of W251N with GlcNAc-biotin (<10%) indicates that the W251N substitution reduces the preference for GlcNAc as the acceptor substrate. This was supported by the difference in their $K_m$ values: 3.5 ± 0.8 mM for N175Q with 4-nitrophenyl-GlcNAc (33) and 226 ± 29.2 mM for W251N with GlcNAc-biotin. As mentioned above, the N175Q mutant retains its transglycosylation activity but lacks hydrolytic activity. We then generated a double mutant, N175Q/W251N. As expected, the double mutant showed the characteristics of both enzymes: similar (maximum) yield with both fucosylated and

**TABLE 4**
**Conservation (and variance) of the amino acid residue at position 251 of Endo-M among GH85 members and their preference for core-fucosylated glycan substrates**

| Enzyme | Alignment[a] | Preference for core fucosylated glycans | Reference |
|---|---|---|---|
| | Trp 251 of Endo-M | | |
| Endo-D | A D N F F A N F **N** W D K A K N D Y 4 0 8 | Yes | (14) |
| Endo-BB | A D E M F L N F **W** W T E D K L A G 3 1 5 | No | (52) |
| Endo-A | A D S M F L N F **W** W R D Q R Q - - 2 7 4 | No | (13) |
| Endo-BH | A D S M F L N F **W** W W N H S Q E R 2 7 5 | No | (51) |
| Endo-CC1 | S S G I F T N Y **W** W Y N D A P Q K 2 6 1 | -[b] | (16) |
| Endo-CC2 | S S G L F T N Y **A** W Y N H F P Q R 2 6 7 | -[b] | (16) |
| Endo-M | T D G I F L N Y **W** W K K E Y P E M 2 5 9 | No | (26, 27) |
| Endo-Om | S D A F F S N Y **W** W N I K N L Q E 2 7 4 | No | (15) |
| Endo-Os | C D G L F S N Y **T** W K A K Y P Q E 2 6 7 | No | (53), pc[c] |
| Endo-CE | C D A I Y L N Y **N** W K D K E L L R 2 3 0 | No | (50) |
| HsENGase | C D G F F T N Y **N** W R E E H L E R 3 1 3 | -[b] | (2) |
| MmENGase | C D G F F T N Y **N** W R E D H L Q R 3 0 5 | -[b] | - |

[a] GenBank™ accession number of amino acid sequences used for alignment are as follows: BAB62042.1 (Endo-D), AAN25135.1 (EndoBB), AAD10851.1 (Endo-A), BAB04504.1 (Endo-BH), XP_001839402 (Endo-CC1), XP_002911817 (Endo-CC2), BAB43869.1 (Endo-M), BAN20080.1 (Endo-Om), BAF17177.1 (Endo-Os), BAB84821.1 (Endo-CE), AAM80487.1 (human ENGase), and BAC33415.1 (*Mus musculus ENGase*).
[b] No available information.
[c] Personal communication with Dr. Yoshinobu Kimura (Okayama University).

afucosylated acceptor substrates but no apparent yield decline during the prolonged incubation time because of the loss of its hydrolytic activity (Fig. 6, *C* and *D*), suggesting that both effects of N175Q and W251N on the reaction are independent of each other. Furthermore, to increase the product yield, we tested the effect of adding DMSO to the transglycosylation reaction. Adding 5 or 10% DMSO in the reaction solution enhanced the transglycosylation yield at 2 h (~20% increase in 10% DMSO compared with the reaction without DMSO), probably due to increased substrate solubility. The effect of donor substrate concentration was also examined using the N175Q/W251N double mutant (Fig. 6*E*). The yields were almost proportionally increased by >22% when 20 mM SG-oxazoline was added, in which the donor/acceptor ratio was 4:1. This result indicated that the Endo-M N175Q/W251N double mutant may be practically useful for synthesizing various core-fucosylated glycoconjugates.

## Discussion

The lack of substrate specificity of wild-type Endo-M for core-fucosylated glycans limits its scope of application for glycoproteins that are core-fucosylated. In the present study, we found that Trp-251 of Endo-M is an important residue that determines its substrate preference for core-fucosylated glycan substrates. Among Trp-251 mutants, the W251N variant showed the highest hydrolytic activity with core-fucosylated glycans and exhibited transglycosylation activity in a reaction mixture with α1,6-fucosyl GlcNAc-biotin as the acceptor. The yield of the transglycosylation product with the fucosylated acceptor substrate reached up to 22% relative to the control reaction. Our findings will facilitate the efficient production of homogeneous glycoforms of various glycoconjugates, including glycopeptides/glycoproteins.

The replacement of a single amino acid residue from tryptophan to asparagine at position 251 of Endo-M changed the substrate preference of this enzyme from afucosylated glycans to core-fucosylated glycans. As shown in Table 4, the asparagine residue at this position is conserved among several GH85 enzymes, including bacterial Endo-D, Endo-CE from nematode (50), and some mammalian ENGases (2). On the other hand, the tryptophan residue is conserved among enzymes such as bacterial Endo-A, Endo-BH from *Bacillus halodurans*

C-125 (51), Endo-BB from *Bifidobacterium longum* DJO10A (BLD_0197) (52), fungal Endo-M, Endo-CC1 (16), and Endo-Om from methylotrophic yeast *O. minuta* (15). Although there appears to be no clear correlation between the Trp/Asn residue and substrate specificity of these enzymes, it is likely that the enzymes with a tryptophan residue at that position do not act on core-fucosylated glycans, whereas the enzymes having an asparagine at that position can act on both types of substrates. There are exceptions, such as fungal Endo-CC2, which has an alanine residue at this position, and plant Endo-Os from rice *Oryza sativa*, which has a threonine residue at this position (53). The tryptophan residues often interact with residues of the glycan substrates and stabilize the binding of the substrate in the subsite. Therefore, we speculate that Trp-251 of wild-type Endo-M may be interacting with the innermost GlcNAc residue of the glycan, possibly due to the proximity between these residues. Fucosylation of the innermost GlcNAc residue might inhibit its interaction. In addition, future elucidation of the three-dimensional structure of the W251N mutant enzyme will give insights into the role of the asparagine residue in its interaction with core-fucosylated glycan residues and reveal the factors that define its substrate preference.

Wang and colleagues have extensively explored the usefulness of endoglycosidases in a convergent chemoenzymatic approach for the *N*-glycosylation of innately core-fucosylated glycoproteins, including the IgG molecule. The Endo-D glycosynthase N322Q mutant is capable of transferring a trimannosyl sugar donor onto an α1,6-fucosyl GlcNAc on IgG (40). The Endo-S glycosynthase D233Q mutant has been used successfully for the conversion of *N*-glycans of the Fc region in IgG (43). Endoglycosidase Endo-F3 from *Elizabethkingia meningosepticum* and its glycosynthase variant have been shown to exhibit transglycosylation activity to synthesize core-fucosylated sialoglycopeptides (41, 42). Recently, Endo-S2 was reported to possess a more relaxed substrate specificity for donor and acceptor substrates with a higher efficiency than Endo-S (46). Efforts to develop an enzyme that acts on various *N*-glycan donor species and various glycoconjugates, including glycoproteins, are successfully expanding the applicability of this chemoenzymatic approach.

By site-directed mutagenesis, we isolated an Endo-M mutant enzyme exhibiting altered substrate specificity compared with wild-type Endo-M. To the best of our knowledge, this is the first report that endoglycosidase substrate specificity is altered by site-directed mutagenesis. This finding will lead to a novel approach for developing new enzymes with broader substrate specificity rather than isolating novel enzymes from nature. The Endo-M W251N variant could act on core-fucosylated glycopeptides derived from IgGs and the native form of human lactoferrin. Although intact IgG molecules may not be as good substrates for W251N as that for Endo-S, glycopeptides and some globular glycoproteins like hLF may be good substrates. This finding can open up new avenues and provide novel options for enzymatic tools in the field of glycobiology. It is one option available for efficient use in convergent chemoenzymatic synthesis of glycoprotein-based therapeutics that have specific glycoforms for enhanced potency.

## Experimental Procedures

*Reagents*—*N*-Acetylglucosaminyl biotin (G0297; GlcNAc-biotin), α1,6-fucosyl-*N*-acetylglucosaminyl biotin (F1021; Fucα1-6GlcNAc-biotin), trimannosyl chitobiosyl biotin ($Man_3$-$GlcNAc_2$-biotin, M3-biotin), and core-α1,6-fucosylated trimannosyl chitobiosyl biotin ($Man_3GlcNAc_2Fuc_1$-biotin, M3F-biotin) were supplied by Tokyo Chemical Industry Co., Ltd. (supplemental Scheme S1 and Fig. S1). PA-oligosaccharide substrates were purchased from TaKaRa Bio Inc. (Otsu, Japan) or Masuda Chemical Industries Co., Ltd. (Takamatsu, Japan). Rituximab (Rituxan®), an anti-CD20 monoclonal antibody, was obtained from Zenyaku Kogyo Co., Ltd. (Tokyo, Japan). $(Neu5Ac_1Gal_1GlcNAc_1)_2Man_3GlcNAc$-oxazoline (SG-oxazoline) was prepared as described previously (36). Human lactoferrin and Endo-$F_3$ were purchased from Sigma-Aldrich. PNGase F was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Endo-S was purchased from New England BioLabs (Ipswich, MA).

*Site-directed Mutagenesis*—Site-directed mutagenesis to generate Endo-M mutant enzymes was performed by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan), according to the procedure prescribed in the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). A pET23b-Endo-M plasmid containing the cDNA fragment of wild-type Endo-M fused with a hexahistidine tag at the C terminus, was used as the template DNA (17). The following primers and their complementary strands were used: 5′-GTGTTTGGTACTTTTTTAGTAGAATGGAATAACCAAATGCATG-3′ (for G125W), 5′-GTAGAAGGAAATAACGCAATGCATGAAATGGAAGCCTTGC-3′ (for Q128A), 5′-GTAGAAGGAAATAACTCAATGCATGAAATGGAAGCCTTGC-3′ (for Q128S), 5′-GACAAATGAAGGAGAAATCCACCACCAGAACCAGCTCACATGG-3′ (for W228H), 5′-CGGATGGTATTTTTTTGAATTATGCGTGGAAAAAAGAATACCCTG-3′ (for W251A), and 5′-CGGATGGTATTTTTTTGAATTATAACTGGAAAAAAGAATACCCTG-3′ (for W251N). For saturation mutagenesis of the Trp-251 residue, a forward primer (5′-CGGATGGTATTTTTTTGAATTATNNSTGGAAAAAAGAATACCCTG-3′) and a reverse primer (5′-CAGGGTATTCTTTTTTTCCASNNATAATTCAAAAAAATACCATCCG-3′) were used. Mutations were confirmed by DNA sequencing.

*Protein Expression in E. coli and Purification of Recombinant Endo-M*—*E. coli* strain BL21 (DE3) was transformed with the plasmid containing mutated Endo-M genes, and the transformants were cultured in Luria-Bertani medium containing 100 mg/liter ampicillin at 19 °C for 38 h to express Endo-M mutants at a basal level. The cells were harvested and lysed in BugBuster Master Mix (Merck Millipore, Darmstadt, Germany) supplemented with 1 mM phenylmethylsulfonyl fluoride, and the cell debris and insoluble materials were removed by centrifugation. The clear supernatant obtained was then loaded onto a 1-ml $Ni^{2+}$-charged His-Trap chelating column (GE Healthcare, Little Chalfont, UK) pre-equilibrated with the binding buffer (20 mM sodium phosphate, pH 7.5, containing 0.5 M sodium chloride and 10 mM imidazole). Unbound proteins were washed out with the binding buffer. Endo-M mutants were eluted with the

elution buffer (20 mM sodium phosphate, pH 7.5, containing 0.5 M sodium chloride and 100 mM imidazole). The eluates were desalted and buffer-changed to 20 mM sodium phosphate, pH 7.2, using an Amicon Ultra filter device (30K, Millipore). Endo-M mutants were further purified by Mono Q anion exchange column chromatography. The enzyme solutions were loaded onto a Mono Q 5/50 GL column (GE Healthcare) pre-equilibrated with 20 mM sodium phosphate, pH 7.2. The elution was carried out using a linear gradient of 20 mM sodium phosphate, pH 7.2, containing 1 M sodium chloride (concentrated up to 50%), in 20 column volumes. The eluates were analyzed by SDS-PAGE/Coomassie Brilliant Blue staining, and the fractions containing purified Endo-M mutant enzymes were combined and concentrated using Amicon Ultra filter devices (30K). The protein concentrations were quantified using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific) and used in the following enzyme assays.

*Enzyme Assays*—Assays for the hydrolytic activity of the Endo-M enzymes on biotinylated oligosaccharides were performed by incubating 3 mM M3-biotin or M3F-biotin (as the substrate) with 20–200 ng of the enzyme in 30 mM sodium phosphate buffer (pH 6.0), at 30 °C. The reaction was stopped by heating at 95 °C for 5 min. The reaction mixtures were analyzed by thin layer chromatography or high performance liquid chromatography, as described below. To examine the relative activity of the mutant enzyme with PA-oligosaccharides as the substrate, 4 mM PA-oligosaccharides were incubated with either 400 ng of wild-type or 1 $\mu$g of W251N mutant enzyme in 20 mM sodium phosphate buffer, pH 6.0, at 30 °C for 20 min. The reactions were stopped by heating at 95 °C for 5 min. The reaction mixtures were analyzed by high performance liquid chromatography.

An assay for the transglycosylation activity of the Endo-M mutant enzyme was performed at 30 °C by incubating 5–20 mM SG-oxazoline (as the glycan donor substrate) with 5 mM Fuc$\alpha$1–6GlcNAc-biotin or GlcNAc-biotin (as the acceptor substrate) and 0.66 $\mu$g of Endo-M mutant enzyme in 25 mM sodium phosphate buffer, pH 6.5. The reaction was stopped by heating at 95 °C for 5 min.

*TLC Analysis*—TLC was performed using silica gel 60-coated aluminum plates (Merck). The samples were spotted on the plates and developed in a solvent (*n*-butanol/acetic acid/water, 3:2:2). To visualize the saccharides by color development, the plate was sprayed with orcinol-sulfuric acid reagent and heated in a toaster.

*HPLC Analysis*—Analytical reversed-phase HPLC was performed on a Waters e2695 separation module with a Cosmosil 5C18-AR-II column (4.6 × 150 mm; Nacalai Tesque, Japan) at 40 °C. The biotinylated oligosaccharides were eluted using an isocratic solvent system (90% solvent A (0.1% trifluoroacetic acid), 10% solvent B (0.1% trifluoroacetic acid in acetonitrile)) at a flow rate of 0.5 ml/min, along with UV monitoring at 214 nm using a Waters 2998 photodiode array detector. For PA-oligosaccharides, reversed-phase HPLC was carried out using a Cosmosil 5C18-AR-II column (4.6 × 150 mm) at 40 °C, and the elution was done using an isocratic solvent system: 96% solvent A (100 mM acetic acid-triethylamine, pH 4.0), 4% solvent B (100 mM acetic acid-triethylamine, pH 4.0, containing 0.5% *n*-butanol) at a flow rate of 1.0 ml/min, accompanied by monitoring of the fluorescence of the pyridylamino group (excitation, 320 nm; emission, 400 nm) using a Waters 2475 multiwavelength fluorescence detector.

*Preparation of Tryptic Glycopeptide/Peptide Mixture of Rituximab and Human Lactoferrin*—Rituximab (800 $\mu$g) or human lactoferrin (200 $\mu$g) was incubated in 40 mM ammonium bicarbonate at 55 °C for 45 min in the presence of 10 mM dithiothreitol. After it cooled down to room temperature, iodoacetamide was added to a final concentration of 30 mM, and the tube was incubated at room temperature for 1 h in the dark. The alkylated glycoprotein was then digested using 5 $\mu$g of sequencing grade modified trypsin (Promega) at 37 °C. The tryptic peptides were dried, reconstituted in 5% acetic acid, and applied onto a C18 cartridge column (Waters) pre-equilibrated with 5% acetic acid, followed by sequential elution with 20% 2-propanol, 5% acetic acid and 40% 2-propanol, 5% acetic acid. The eluates were evaporated to dryness in a SpeedVac concentrator.

*Release of N-Glycans from Glycopeptides by Peptide:N-Glycanase F or Endoglycosidases*—For *N*-glycans of rituximab, the glycopeptide/peptide mixture equivalent to 200 $\mu$g of rituximab was treated overnight with 1 unit of PNGase F, 4 $\mu$g of wild-type Endo-M, 4 $\mu$g of W251N mutant Endo-M, and Endo-S (100 units). For *N*-glycans of lactoferrin, the glycopeptide mixture from 200 $\mu$g of lactoferrin was treated with 1 unit of PNGase F. These reaction mixtures were evaporated in a SpeedVac concentrator, reconstituted in 5% acetic acid, and applied onto a C18 cartridge column (Waters). The released *N*-glycans were collected in the flow-through fractions and lyophilized. Permethylation of the endoglycosidase-released *N*-glycans was carried out, according to the standard method (54), followed by MALDI-TOF/MS and MALDI-TOF/TOF-MS analyses, as described below.

*Enzymatic Treatment of Human Lactoferrin*—Ten micrograms of hLF was treated overnight with Endo-S (100–1,000 units), wild-type Endo-M (2 $\mu$g), or the W251N variant (2 $\mu$g). Aliquots of reaction mixtures were resolved by SDS-PAGE on 7.5% acrylamide gel and visualized by Coomassie Brilliant Blue staining. The released *N*-glycans were purified, as described above, and analyzed by MALDI-TOF/MS.

*MALDI-TOF Mass Spectrometry Analysis*—The permethylated glycan samples were reconstituted in 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in 50% methanol) and then analyzed by MALDI-TOF/MS or MALDI-TOF/TOF-MS using UltrafleXtreme (Bruker Daltonics, Billerica, MA) in the positive ion mode.

*Author Contributions*—T. Katoh, T. Katayama, and K. Y. designed the study and wrote the manuscript. T. Katoh, T. Katayama, Y. T., Y. N., J. K., and Y. M. performed the experiments and discussed the data. All authors reviewed the results and approved the final version of the manuscript.

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