Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation

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Monoclonal antibodies (mAbs) have been developed as therapeutics, especially for the treatment of cancer, inflammation, and infectious diseases. Because the glycosylation of mAbs in the Fc region influences their interaction with effector cells that kill antibody-targeted cells, and the current method of antibody production is relatively expensive, efforts have been directed toward the development of alternative expressing systems capable of large-scale production of mAbs with desirable glycoforms. In this study, we demonstrate that the mAb trastuzumab expressed in glycoengineered *P. pastoris* can be remodeled through deglycosylation by endoglycosidases identified from the Carbohydrate Active Enzymes database and through transglycosylation using glycans with a stable leaving group to generate a homogeneous antibody designed to optimize the effector functions. The 10 newly identified recombinant bacterial endoglycosidases are complementary to existing endoglycosidases (EndoA, EndoH, EndoS), two of which can even accept sialylated tri- and tetraantennary glycans as substrates.

glycoengineered antibodies | *Pichia* | trastuzumab | endoglycosidase | Fc glycosylation

Monoclonal antibodies (mAbs) have been developed as effective medicines for the treatment of various diseases. Trastuzumab (Herceptin), for example, is an antibody that targets human epidermal growth factor receptor 2 (HER2) and has been used for the treatment of breast cancer with HER2 overexpression (1, 2), and has become one of the most effective targeted therapeutics (3–5). One of trastuzumab's mechanisms of action (6) is via antibody-dependent cellular cytotoxicity (ADCC): the activation of natural killer cells to initiate lysis of cancer cells that are bound to trastuzumab. Trastuzumab also inhibits the formation of p95, a truncated membrane-bound fragment that results from cleavage of the extracellular domain of HER2 and has in vitro kinase activity. In addition, trastuzumab inhibits the phosphoinositide 3-kinase pathway, which is activated by overexpression of HER2 (7).

Most therapeutic mAbs are of the IgG class and contain a glycosylation site in the Fc region at position 297. The glycan on this glycosylation site plays a critical role in effector functions (8, 9), including complement activation (leading to complement-dependent cytotoxicity) and ADCC through interaction with Fc receptors (FcγRs) FcγRI, FcγRIIA, FcγRIIIA, and B lymphocyte activation via FcγRIIB (10, 11). Therefore, engineering the Fc glycan of IgG1 to modulate IgG/FcγRIIIA interactions has become a major goal over the last decade after a number of reports showing that FcγRIIIA plays an important role in the efficacy of therapeutic mAbs (12). However, the antibodies produced from mammalian cells exist as heterogeneous glycoforms. It is thus necessary to develop mAbs with well-defined Fc glycans to improve their safety and efficacy. Toward this goal, it has been reported that removal of the core fucose residue increases Fc interaction with FcγRIIIA receptor, and thus enhances the ADCC activity of IgG (13, 14). However, all the antibodies described here were still heterogeneous even when a specific glycan structure was enriched through pathway engineering. To understand the effect of Fc glycans on antibody's functions, homogeneous antibodies with well-defined glycan structures are needed (15–17). Recently, our group demonstrated that the biantennary *N*-glycan with two terminal α-2,6-linked sialic acids (α2,6-SCT) at position 297 of the Fc region is an universal and optimized structure for the enhancement of ADCC, complement-dependent cytotoxicity, and anti-inflammatory activities (17). In another study, we used an effective fucosidase to remove the core-fucose to increase the binding affinity between mAb and FcγR receptors (18).

Mammalian cell lines such as Chinese hamster ovary cells are commonly used as hosts for mAb production, but the process is relatively expensive (estimated at $300–$3,000/g) (19). Therefore, alternative platforms using plant and microbial expression systems (20–22) have been developed, of which plant-produced trastuzumab was shown to be as functional as the Chinese hamster ovary-produced product (23, 24). Of these alternative platforms, yeast-based approaches are regarded as a compelling alternative to mammalian cell culture because of their possibly higher titers, low-cost and scalable fermentation process, and low risk for human pathogenic virus contamination. In addition, the glycosylation pathway in yeast can be engineered to avoid fungal-type glycosylation and enable the production of antibodies with glycosylation profiles similar to that found in humans without core fucosylation in the *N*-liked glycans. However, the wild-type methylotrophic yeast *Pichia pastoris* has the glycosylation pathway that produces glycoproteins with high-mannose-type glycans (25), which reduce the in vivo half-life and compromise therapeutic function, or are even immunogenic. Manipulation of the

Significance

The carbohydrate components of glycoproteins are known to affect the structure and function of glycoproteins, and thus it is important to develop effective tools to manipulate and optimize the glycan components of glycoproteins with therapeutic significance. This study demonstrates the discovery of endoglycosidases for the remodeling of glycans on Herceptin, a monoclonal antibody used in the treatment of breast cancer, to optimize its effector functions, especially the antibody-dependent cellular cytotoxicity (ADCC). In addition, a method has been developed for the expression of antibodies from yeast to enable the large-scale synthesis of antibodies for further manipulation of the glycan moiety, using endoglycosidases and stable transglycosylation donors to prepare a homogeneous glycoform with optimized effector functions.

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glycosylation pathway in *P. pastoris* to produce glycoproteins with *N*-glycosylation profiles similar to the ones from human has been pursued, but the products are still heterogeneous (26).

To manipulate the glycan moiety of antibody, we investigate the glycoside hydrolases (GHs) from the Carbohydrate Active Enzymes database (www.cazy.org) (27). Glycoside hydrolases include a widespread group of enzymes that hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (28). At this time, 145 families of GHs were described, among which 54 were grouped in 15 clans of related families (29, 30). Three endoglycosidase GH families, GH18, GH20, and GH85, display a hydrolysis mechanism with net retention of configuration.

Of the endoglycosidases known as *endo*-β-*N*-acetyl-glucosaminidases (ENGases) used in glycoprotein remodeling, all have different tolerances to the neighboring residues around the cleavage site (31). For instance, endoglycosidase H (EndoH), from *Streptomyces plicatus* (*griseus*), is specific for the high mannose/hybrid-type glycans; EndoM, from *Mucor hiemalis*, is specific for high mannose/hybrid-type and biantennary complex-type glycans; and EndoF1/F2/F3, from *Elizabethkingia meningoseptica*, are specific for high mannose/hybrid-type, biantennary complex-type, and bi/triantennary complex-type glycans, respectively (31). EndoE, from the human pathogen *Enterococcus faecalis*, encoded by *ndoE*, consists of an α-domain with a family GH18 motif and a β-domain similar to family GH20 glycosyl hydrolases, which can degrade the *N*-linked glycans of human RNase B to acquire nutrients. Phylogenetic analysis of EndoE indicates that the α-domain is related to human chitobiases, and the β-domain is related to bacterial and human hexosaminidases. Recombinant expression of full-length EndoE or EndoE-α shows that EndoE-α hydrolyzes the glycan on human RNase B, whereas EndoE-β hydrolyzes the conserved glycan on IgG (32). EndoS is a secreted glycoside hydrolase produced by the group A *Streptococcus pyogenes*, which shows specific endoglycosidase activity against human IgG (33). Specifically, EndoS cleaves the complex-type β1→4 linkage within the di-*N*-acetylchitobiose core of the *N*-linked glycan present at Asn297 of the IgG CH2 domain (33). It also shows the specificity for complex biantennary-type glycans, and as such, has gained interest for use as a specific transglycosidase for the chemoenzymatic synthesis of antibody glycoforms (16, 34). ENGase mutants with reduced glycosidic activity (called glycosynthase, such as EndoS-D233A and EndoS-D233Q) generated by site-directed mutagenesis showed remarkable transglycosylation efficiency capable of transferring complex type *N*-glycans from activated glycan oxazolines to deglycosylated intact antibodies without product hydrolysis. These two mutants acted efficiently on both the core-fucosylated and nonfucosylated GlcNAc-Fc domain of intact antibodies to provide various defined IgG glycoforms (16). In 2013, Sjögren and coworkers (35, 36) reported EndoS2, an endoglycosidase from *Streptococcus pyogenes* of serotype M49, with broader glycan substrate specificity in Fc deglycosylation than EndoS. Moreover, Wang's group found that EndoS2 possessed potent transglycosylation activity, and

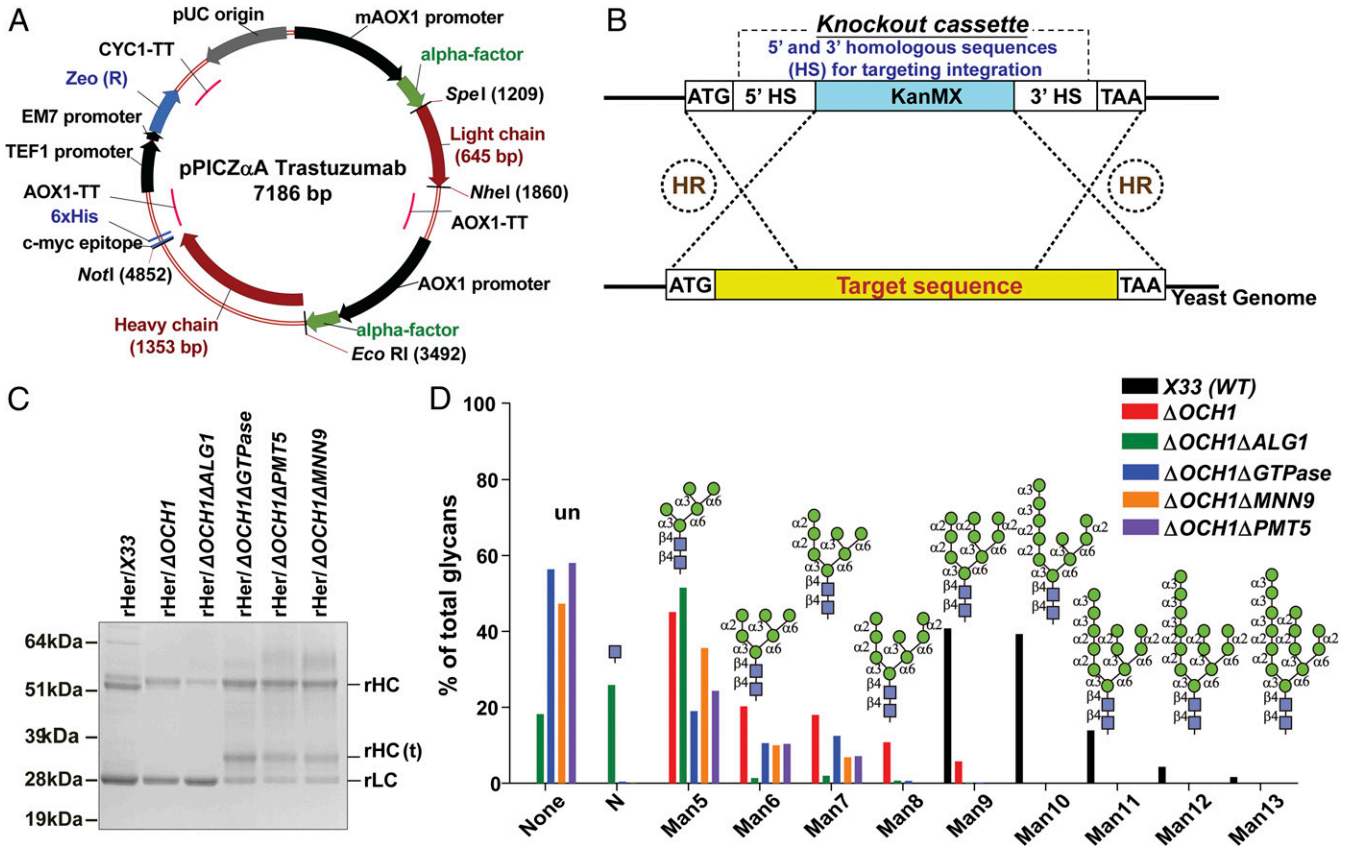

Fig. 1. Construction and expression of recombinant trastuzumab in *P. pastoris* and characterization of glycans on purified glycoproteins using liquid chromatography–mass spectrometry analysis. (*A*) Construction of expression plasmid. (*B*) KanMX replacement cassette for homologous recombination of yeast chromosomal integration module. (*C*) Characterization of recombinant trastuzumabs produced by WT and YKO *P. pastoris* strains by using SDS/PAGE. (*D*) Glycan profiling of recombinant trastuzumabs produced by WT and YKO yeast strains. 5′HS/3′HS, 5′/3′-end homologous sequence; 6xHis, the polyhistidine tag; Δ, gene deletion; heavy chain, the heavy chain OR of trastuzumab; KanMX, kanamycin (G418) resistance gene; Light chain, the light chain OR of trastuzumab; mAOX1 promoter, modified alcohol oxidase 1 promoter region; rHC, recombinant heavy chain; rHC(t), truncated form of recombinant heavy chain; rLC, recombinant light chain; TT, the alcohol oxidase 1 transcriptional terminator region; Zeo(R), zeocin resistance gene.

the systematic site-directed mutagenesis led to the discovery of several glycosynthase mutants, including D184M and D184Q, that showed remarkable transglycosylation activity using glycan oxazolines (37). The EndoS2 glycosynthases were capable of transferring three major types (complex, high-mannose, and hybrid) of *N*-glycans for antibody glycosylation remodeling (36, 37). However, none of the existing ENGases is capable of accepting tetraantennary glycans or sialylated triantennary glycans as substrates, and the oxazolines are relatively reactive and exhibit nonenzymatic glycosylation to produce byproducts, and thus may not be ideal for large-scale operation (38, 39).

In this study, we report an expressing platform, strain engineering, and production processes, using yeasts for antibody production. In addition, we discovered endoglycosidase enzymes that are capable of efficiently hydrolyzing the heterogeneous glycans of the recombinant trastuzumab to form a monoglycosylated IgG with GlcNAc attached to the Asn group, which is further used for the preparation of homogeneous trastuzumab with well-defined glycan structures using glycans with a stable leaving group as transglycosylation donors. Some ENGases can also accept sialylated tri- and tetraantennary glycans with stable leaving groups as substrates. These methods of homogeneous mAb production overcome the drawbacks mentioned here and open a window for glycoengineering.

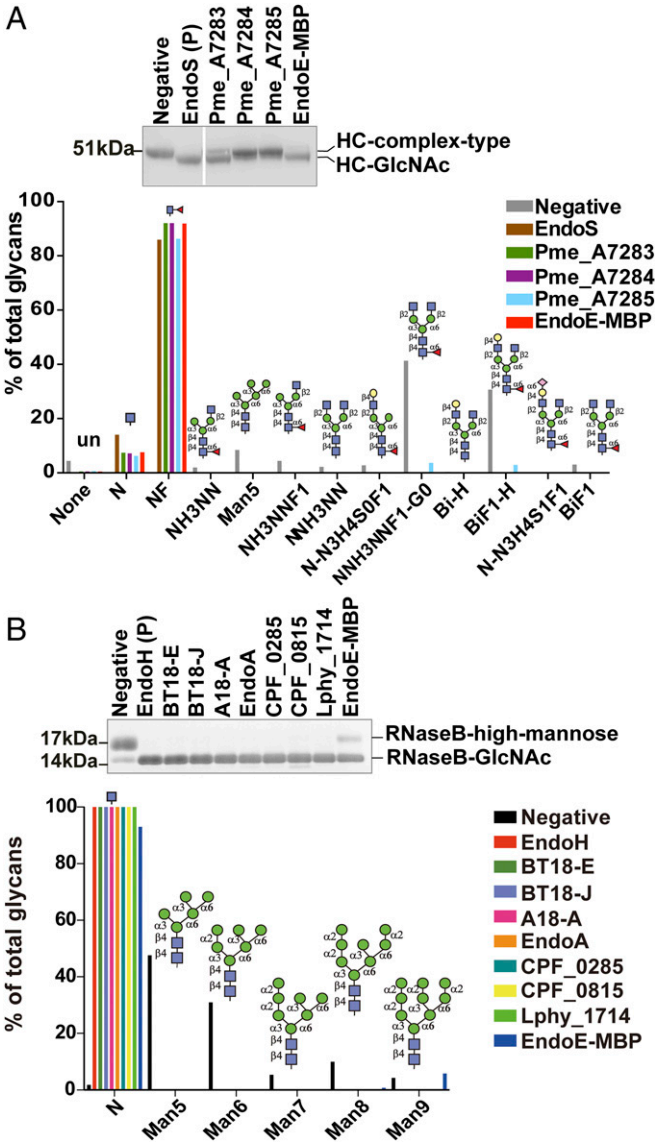

Fig. 2. ENGase catalyzed hydrolysis of complex and high-mannose type glycoproteins. (*A*) Characterization of glycan hydrolysis activities on Herceptin. (*B*) Characterization of glycan hydrolysis activities on RNaseB. (*Upper*) Western blotting. (*Lower*) Glycan MS.

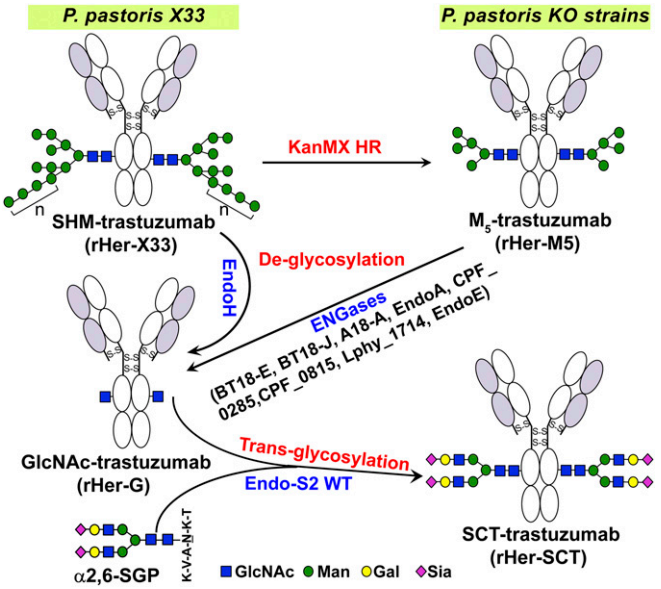

Fig. 3. Glycosylation remodeling of recombinant trastuzumab to prepare homogeneous glycoforms with well-defined glycan of sialyl-2,6-linkage for optimal effector functions. GlcNAc-trastuzumab, recombinant trastuzumab with single GlcNAc in the Fc region; M_5-trastuzumab, recombinant trastuzumab with Man5 glycans in the Fc region as a major glycoform; SCT, sialylated biantennary complex type glycan; SGP, sialylated biantennary complex type glycopeptide; SHM-trastuzumab, recombinant trastuzumab with super high mannose glycans in the Fc region.

Results and Discussion

Construction of Plasmids in Yeast Strains. The complete trastuzumab (DrugBank Accession Number: DB00072) heavy chain and light chain coding sequences were optimized to *P. pastoris* codon. All genes were obtained via cDNA synthesis and were ligated into the modified pPICZαA vector with N-terminal α-factor for protein secretion. The light chain gene was inserted between the modified AOX1 promoter and AOX1 transcriptional terminator with fused α-factor signal peptide at the N-terminal and the fused poly-histidine tag at the C-terminal, and linked the heavy chain expression cassette in the same expression vector (Fig. 1*A*). The yeast strain *Pichia pastoris* ×*33* is a wild-type strain that is applied to the selection of Zeocin-resistant expression vectors. *Pichia* GlycoSwitch strain *SuperMan5* is an *OCH1* disruption strain available from BioGrammatics and mainly expresses the target protein with a mannose-5 structure at *N*-glycosylation sites. The double-gene knockout strains were derived from *SuperMan5* by the homologous recombinant chromosomal integration method (Fig. 1*B*). We used a PCR-based gene deletion strategy to generate a start-codon to stop-codon deletion of each of the ORFs in the yeast genome. Each gene disruption was replaced with a KanMX module and uniquely tagged with two 20–40 mer sequences (primers used for fragment assembly and amplification of genomic DNA were listed on *SI Appendix*, Table S1). The yeast knockout strains (YKOs) were identified by PCR, using genomic DNA as templates (*SI Appendix*, Fig. S1). The glycoengineered *P. pastoris* strains were used for the production of full-length mAbs.

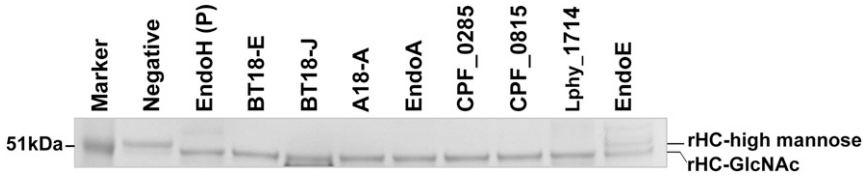

Fig. 4. Characterization of selected ENGases with regard to the deglycosylation activity on recombinant *rHer/ΔOCH1*. Two micrograms purified recombinant *rHer/ΔOCH1* was digested with 100 ng ENGase at 37 °C for 4 h and profiled by SDS/PAGE.

Expression, Purification, and Characterization of Recombinant Trastuzumab Expressed in *P. pastoris*. Intact recombinant trastuzumab was expressed in *P. pastoris* ×*33*, *SuperMan5*, and YKOs in yeast culturing (BMGY) and inducing (BMMY) medium, and labeled as *rHer/X33*, *rHer/ΔOCH1*, *rHer/ΔOCH1ΔALG1*, *rHer/ΔOCH1ΔGTPase*, *rHer/ΔOCH1ΔPMT5*, and *rHer/ΔOCH1ΔMNN9*. Secreting proteins were collected after methanol induction for 48 h with shaking in an incubating box at 23 °C. The recombinant trastuzumab antibodies were captured from the culture supernatant by *rProtein A* and *rProtein L* affinity chromatography and characterized by using SDS/PAGE or Western blotting under reducing conditions (Fig. 1*C*). The yield of recombinant trastuzumab from *Pichia* expression and purification was estimated to 0.5 g/L. Purified recombinant trastuzumabs were composed of fully assembled antibody including double heavy and light chains, and the quality of the antibody profile was comparable to that of commercial trastuzumab. Differences of glycans on commercially available trastuzumab (Herceptin), *rHer/X33*, and *rHer/ΔOCH1* showed differences in molecular weight (*SI Appendix*, Fig. S2). To determine the glycan sequences of these recombinant trastuzumab from different sources, antibodies were treated with trypsin to cleave the protein to peptides, and then the digested products were analyzed by MS with two PeptideCutter TKPREEQYNSTYR and EEQYNSTYR nearby Asn297 of the CH2 domain. The resulting *rHer/X33* and *rHer/ΔOCH1* strains have mannose type *N*-glycans compared with Herceptin with complex type *N*-glycans (Figs. 1*D* and 2*A*). *N*-glycans of *rHer/X33* were highly heterogeneous with hypermannosylation (Man9–Man13). However, the *N*-glycans of *rHer/ΔOCH1* were distributed from Man5 to Man9, and mainly $Man_5GlcNAc_2$ (~42–45% of total glycans were Man5), with reduced hypermannosylated glycoprotein profiling (Fig. 1*D*). Furthermore, the glycan compositions of recombinant trastuzumab of double-mutation strains (YKOs) were shorter and simpler than that from the wild-type and single-mutated strains (Fig. 1*D*). More important, the *Pichia*-produced trastuzumab was 100% nonfucosylated in the Fc domain. These results indicated that disruption of *OCH1*, *ALG1*, *GTPase*, *MNN9*, and *PMT5* genes in the glycosylation process successfully reduced the capability of hypermannosylation, and our GlycoDelete engineering strategy could produce recombinant trastuzumab with less heterogeneity of *N*-glycans.

Expression of a Library of Bacterial Endoglycosidases and Characterization of Their Deglycosylation Activity on Glycoproteins. To identify glycosidases for glycoprotein deglycosylation, a panel of known and putative *endo*-β-*N*-acetylglucosaminidase (Enzyme Commission 3.2.1.96) and β-hexosaminidases (Enzyme Commission 3.2.1.52) ORFs (putative *ndo* genes) from the Carbohydrate Active Enzymes database glycoside hydrolase family GH18, GH20, and GH85 (listed in *SI Appendix*, Table S2) were cloned into pET28a vector and expressed in *Escherichia coli BL21*. The substrate specificity of recombinant endoglycosidases (ENGases) was first screened by use of commercial Herceptin and RNase B (*SI Appendix*, Fig. S3). Of the 35 recombinant ENGases, four (Pme_A7283, Pme_A7284, Pme_A7285, and EndoE) were found to have the glycosidic activity on complex type *N*-glycans (Fig. 2*A*), and eight (BT18-E, BT18-J, A18-A, EndoA, CPF_0285, CPF_0815, Lphy_1714, and EndoE) were found to have the activity on the high-mannose type (Fig. 2*B*). The glycan hydrolysis activities of screened endoglycosidases were also characterized by a glycan microarray, which was presented with multiantennary complex-type and mannose-type *N*-glycan structures containing the C5-amine linker printed on NHS-activated glass slides through amide bond formation (*SI Appendix*, Fig. S4). Of the ENGases screened, several were capable of hydrolyzing the substrates at the desired bond, and two could even accept the sialylated tri- and tetraantennary glycans. As part of our program aiming to develop efficient enzymatic deglycosylation methods for glycoprotein remodeling (Fig. 3), we turned our attention to recombinant ENGases that are capable of hydrolyzing the *N*-glycans of recombinant *rHer/ΔOCH1* by cleaving the β-1,4-glycosidic bond of the chitobiose core (Fig. 4). Eight recombinant ENGases were found to have efficient deglycosylation ability to give the corresponding Fc with only one nonfucosylated GlcNAc monosaccharide (named *rHer/ΔOCH1-GlcNAc*) at the glycosylation site (N297). The deglycosylated *rHer/ΔOCH1-GlcNAc* was then purified by *rProtein A* and used for the transglycosylation process.

Deglycosylation Activity of EndoE for Cleavage of High-Mannose Glycans on Yeast-Produced Recombinant Trastuzumab. To know the deglycosylation activity of EndoE and the site-directed mutagenesis mutants of the EndoE-α domain, recombinant trastuzumab *rHer/ΔOCH1* was used as the substrate for incubation with the *E. coli*-expressed EndoE-His fusion proteins. WT EndoE-MBP, EndoE-αWT,

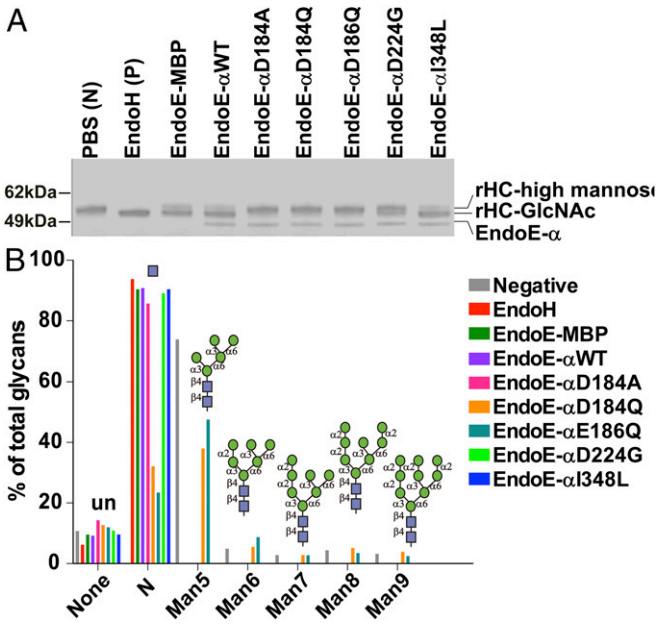

Fig. 5. EndoE-catalyzed deglycosylation of high-mannose glycans on recombinant *rHer/ΔOCH1*. (*A*) Characterization of EndoE-treated *rHer/ΔOCH1*. Two micrograms purified *rHer/ΔOCH1* was digested with 100 ng EndoE at 37 °C for 4 h and profiled by SDS/PAGE. (*B*) Characterization of EndoE-treated *rHer/ΔOCH1* by using liquid chromatography–mass spectrometry. The ratio showed in the *y* axis was relative glycan amount on the Fc region of recombinant *rHer/ΔOCH1*.

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EndoE-αD224G, and EndoE-αI348L were shown to have a competitive deglycosylating activity (compared with EndoH) on *rHer/ΔOCH1* to give a homogeneous glycoprotein with only one GlcNAc in the Fc region. However, EndoE-αD184Q and EndoE-αE186Q mutants lost the deglycosylating activities on mannose-type glycans, indicating that Asp184 and Glu186 of EndoE are important for deglycosylation (Fig. 5). These results confirm the remarkable Fc-glycan hydrolyzing activity of EndoE on intact IgG, suggesting its usefulness in the first step of glycosylation remodeling of mAbs.

Synthesis of Homogeneous Trastuzumab. Previously, we identified a universal glycan that can be successfully conjugated to the GlcNAc-Asn297 site on the Fc-CH2 domain of antibodies to enhance the ADCC, complement-dependent cytotoxicity, and anti-inflammatory activities (17). Although EndoS2 mutants generated remarkable transglycosylation activity using glycan oxazolines without apparent product hydrolysis activity (37), nonenzymatic byproducts were generated with an increase in incubation time (*SI Appendix*, Figs. S5 and S6). To reduce the byproduct formation generated from the oxazoline, we used EndoS2 WT and a stable glycan donor α2,6-linked sialylglycopeptide (SGP) for the preparation of homogeneous antibody from a mixture of trastuzumab glycoforms prepared from yeast (Fig. 6). EndoS2 WT was shown to exhibit a time-dependent transglycosylation activity on *trastuzumab-GlcNAc* (Her-G) to produce the homogeneous trastuzumab bearing with α2,6-SCT (named Her-SCT) at the Fc region, and the yield of fully glycosylated trastuzumab was estimated to be 80% (Fig. 6). This is a landmark for the preparation of therapeutic antibodies using the yeast expression system and stable glycan donors to avoid nonenzymatic byproduct (*SI Appendix*, Figs. S7 and S8) formation caused by the use of relatively reactive oxazolines as substrates.

Glycoengineered Trastuzumab with Strong FcγRIIIA Binding Affinity. To determine the effect of glycan variants among *Pichia*-produced trastuzumab (rHer-Man5), glycoengineered trastuzumab (rHer-SCT), and Herceptin on human FcγRIIIA receptor binding, we analyzed the on-rate (k_{on}), and off-rate constant (k_{off}) and dissociation constant K_D by biolayer interferometry (Table 1). The data showed that the recombinant rHer-M5 and the glycoengineered rHer-SCT had better binding affinities (higher k_{on}, similar k_{off}, and lower K_D) to FcγRIIIA than Herceptin, consistent with our previous results analyzed by surface plasma resonance (17). These results indicated that the heterogeneous glycan compositions on IgG definitely reduced the binding affinity to FcγRIIIA.

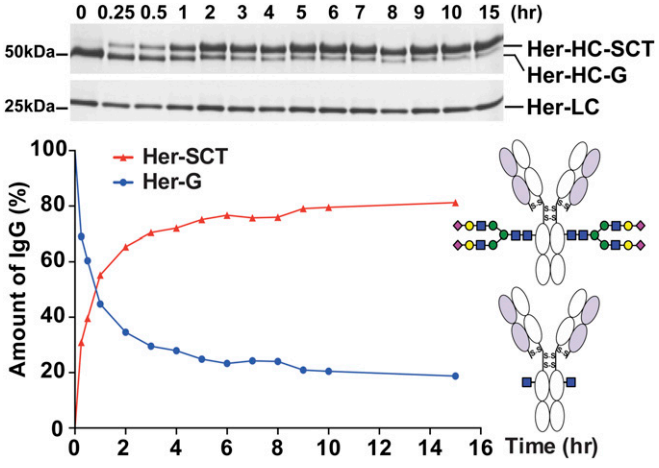

Fig. 6. EndoS2 (WT) catalyzed transglycosylation of trastuzumab. Transglycosylation efficiency was evaluated by SDS/PAGE analysis (*Upper*). The ratio of protein bands on SDS/PAGE was calculated by ImageQuant TL software and presented as the amount of IgG (%) in the *y* axis of the lower plot. G, GlcNAc; HC, heavy chain; Her, trastuzumab; LC, light chain; SCT, α2,6-sialylated biantennary complex type glycan.

Table 1. Biolayer interferometry analysis of the interaction between FcγRIIIA and trastuzumab with different glycoforms

Sample ID and concentration (nM)*	k_{on} ($M^{-1}\cdot s^{-1}$)	k_{off} (s^{-1})	K_D (M)
Herceptin			
50	4.04E+05	6.42E−03	1.59E−08
100	2.83E+05	9.23E−03	3.27E−08
200	1.78E+05	1.09E−02	6.13E−08
400	1.70E+05	8.09E−03	4.77E−08
Grouped	**2.78E+05**	**8.41E−03**	**3.03E−08**
rHer-M5 (*rHer/ΔOCH1*)			
1	9.92E+05	8.03E−03	8.09E−08
5	4.50E+06	3.28E−03	7.29E−10
10	2.60E+06	2.47E−03	9.48E−10
20	1.07E+06	2.02E−03	1.89E−09
Grouped	**2.19E+06**	**2.31E−03**	**1.05E−09**
rHer-SCT			
1	2.96E+07	9.02E−03	3.05E−10
5	3.39E+06	2.32E−03	6.84E−10
10	1.16E+06	3.26E−04	2.82E−10
20	9.24E+05	1.62E−03	1.75E−09
Grouped	**1.16E+06**	**1.51E−03**	**1.31E−09**

k_o, the association rate constant for IgG binding to FcγRIIIA; k_{off}, the dissociation rate constant; K_D, the equilibrium dissociation constant, a ratio of k_{off}/k_{on}, K_D and binding affinity are inversely related; rHer-M5, recombinant trastuzumab with Man5 glycans on the Fc region as major glycoform; rHer-SCT, recombinant trastuzumab with biantennary *N*-glycan with two terminal α-2,6-linked sialic acids on the Fc region.

The association and dissociation phases were recorded for 360 and 300 s, respectively, for every analyte concentration. All steps were performed at 30 °C with an agitation speed of 1,000 rpm on the 96-well plate mode of Octet HTX System (FortéBio). The sensorgrams were measured on an Octet HTX System (FortéBio) and referenced against the buffer reference signal by the Data Analysis Software 9.0.0.10 (FortéBio). The sensorgrams with the concentration signals of each association and dissociation phase were combined (shown in bold grouped values) and processed with referencing for background subtraction and fitted to a 1:1 binding model.

*Herceptin showed better sensorgrams in higher working concentration.

Conclusion

mAbs and their derivatives have been developed for the diagnosis and treatment of various diseases (40). However, many antibody derivatives can only be generated in relatively expensive mammalian cell culture systems, mainly because of the lack of controlled glycosylation (41, 42). In the past years, yeasts have been used for the expression of recombinant antibodies, where glycoengineering is also poised to play a role (43). Previous studies showed that glycoengineered *Pichia*-produced trastuzumab and Chinese hamster ovary-produced trastuzumab displayed similar in vitro biological functions, in vivo antitumor efficacy, and pharmacokinetics in both mice and nonhuman primates (44). Furthermore, using glycoengineering to produce products with well-defined glycans is key to warranting future success (45). In this study, we used the genome-edited *P. pastoris* strain *ΔOCH1* that mainly produces the $Man_5GlcNAc_2$ structure for further glycosylation-gene-editing and production of mAb with an enriched population of high-mannose glycoforms. A number of bacterial ENGases capable of efficient removal of the heterogeneous glycans on the recombinant trastuzumab were identified and used for the production of mono-GlcNAc trastuzumab, which was further converted to homogeneous trastuzumab with well-defined glycan structures using a stable glycan substrate (SGP) as transglycosylation donor. Our study showed that EndoS2 can accept SCT-peptide and complex bi- and triantennary glycans with sialic acids at the

nonreducing end. In addition, based on the glycan array analysis, EndoE and A7283 can even accept tri- and tetraantennary glycan substrates with sialic acid at the nonreducing end and C5-NHR at the reducing end (*SI Appendix*, Fig. S4). This work paves the way to further investigate the effect of multiantennary glycans on antibody's functions and to develop a better transglycosylation reaction on large scales.

Experimental Procedures

Recombinant trastuzumab was expressed in *P. pastoris* (*ΔOCH1* strain), glycoengineered by using the recombinant ENGases (BT18-E, BT18-J, A18-A, EndoA, CPF_0285, CPF_0815, Lphy_1714, or EndoE), a stable glycan donor α2,6-linked SGP, and EndoS2-WT to give homogeneous trastuzumab with α2,6-SCT on the Fc region. The binding affinity between homogeneous trastuzumab and FcγRIIIA was performed by biolayer interferometry. Detailed materials and methods are in the *SI Appendix*, including plasmid construction, enzyme expression, activity analysis of recombinant ENGases, antibody expression in yeast, antibody purification and glycoengineering, and binding analysis of homogeneous antibodies.

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