Glycan Remodeling of Human Erythropoietin (EPO) Through Combined Mammalian Cell Engineering and Chemoenzymatic Transglycosylation

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ABSTRACT: The tremendous structural heterogeneity of N-glycosylation of glycoproteins poses a great challenge for deciphering the biological functions of specific glycoforms and for developing protein-based therapeutics. We have previously reported a chemoenzymatic glycan remodeling method for producing homogeneous glycoforms of N-glycoproteins including intact antibodies, which consist of endoglycosidase-catalyzed deglycosylation and novel glycosynthase-catalyzed transglycosylation, but its application to complex glycoproteins carrying multiple N-glycans remains to be examined. We report here site-selective chemoenzymatic glycosylation remodeling of recombinant human erythropoietin (EPO) that contains three N-glycans. We found that the generation of a HEK293S GnT I knockout FUT8 overexpressing cell line enabled the production of an unusual Man9GlcNAc2Fuc glycoform, which could be converted to a core-fucosylated GlcNAc-EPO intermediate acceptor for enzymatic transglycosylation. With this acceptor, homogeneous sialylated glycoform or azide-tagged glycoform were produced using the glycosynthase (EndoF3-D165A) catalyzed transglycosylation. Interestingly, a remarkable site-selectivity was observed in the transglycosylation reactions, leading to the introduction of two N-glycans selectively at the Asn-38 and Asn-83 sites, which was confirmed by a detailed MS/MS analysis of the transglycosylation product. Finally, a different N-glycan was attached at the third (Asn-24) site by pushing the enzymatic transglycosylation with a distinct glycan oxazoline, achieving the site-selective glycosylation modification of the protein. This study represents the first example of site-selective chemoenzymatic glycan engineering of complex glycoproteins carrying multiple N-glycans.
ribonuclease B. In addition, it has also been successfully applied to the glycan remodeling of therapeutic IgG antibodies due to the discovery of the antibody-specific glycosynthases derived from Endo-S and Endo-S2. Nevertheless, the application of this method for producing homogeneous glycoproteins with more complex glycosylation patterns remained to be tested. The potential challenges include the availability of endoglycosidases for efficient deglycosylation of various N-glycans (especially those highly branched N-glycans) from complex glycoproteins, the efficiency of glycosynthase-catalyzed transglycosylation, the limitation in substrate specificity for transglycosylation, and the site selectivity of glycosylation remodeling in a multiply glycosylated protein. In this paper, we report a combined approach to chemoenzymatic glycosylation remodeling of human erythropoietin (EPO). EPO is a therapeutic glycoprotein that is widely used for the treatment of anemia by boosting red blood cell production. It is a glycoprotein of 166 amino acid residues that carries tremendously heterogeneous N-glycans (bi-, tri-, and tetra-antennary with or without sialic acid) at the three conserved N-glycosylation sites (Asn24, Asn38, and Asn83). It has been demonstrated that proper glycosylation, particularly sialylation, of EPO is critical for the serum half-life of EPO. Recently, total chemical synthesis of homogeneously glycosylated EPO carrying one to three sialylated N-glycans has been achieved via native chemical ligation of synthetic peptides and glycopeptides. Those endeavors have demonstrated the power of chemical methods in the synthesis of homogeneous glycoproteins. Nevertheless, the complexity and multistep manipulations including the construction of complex glycopeptide building blocks, the often low efficiency in native chemical ligation, and the folding of synthetic glycoproteins still pose great challenges in producing significant amounts of pure glycoproteins. We have recently demonstrated that expression of EPO in an engineered HEK293 cell line with overexpression of FUT8 (HEK293S GnT I↑FUT8) resulted in the production of an unusual, fully fucosylated Man5GlcNAc2 glycoform of EPO (EPO-M5F). We report in this paper that the fucosylated Man5GlcNAc2 glycoform of EPO could serve as an excellent starting material for endoglycosidase-catalyzed glycan remodeling to provide sialylated or azide-tagged glycoforms of EPO. Interestingly, we observed a remarkable site-selectivity of the EndoF3-D165A catalyzed transglycosylation, leading to selective transfer of two sialylated N-glycans at the Asn38 and Asn83 sites. This unusually high site-selectivity allows introduction of a different N-glycan, an azide-tagged N-glycan, at the third (Asn24) glycosylation site, leading to site-selective glycan remodeling of EPO.

### RESULTS AND DISCUSSION

#### Inefficient Glycan Remodeling of Complex Type and Nonfucosylated High-Mannose Type Recombinant EPO by Existing Endoglycosidases

The goal for this study is to perform glycan remodeling of recombinant EPO to provide homogeneous glycoforms. EPO protein contains three N-glycosylation sites and one O-glycosylation site. The EPO used in the present research contains a mutation in the S126 O-glycosylation site (serine to valine), as previous studies indicated that the O-glycosylation was not essential for either
stability or bioactivity of EPO.30 Our initial attempt to directly perform glycan remodeling of recombinant EPO expressed from HEK293T cells met with little success. First, we tried to use endoglycosidase to treat complex type EPO to generate a GlcNAc-EPO or Fucα1,6GlcNAc-EPO acceptor. We found that the EPO expressed from HEK293T carries mainly fucosylated tetra-antennary complex type N-glycans (Figure S1, Supporting Information). Treatment of the complex type EPO with Endo-F3 led to no measurable cleavage of N-glycans from the recombinant EPO, as assessed by SDS-PAGE analysis (data not shown). The treatment of the recombinant EPO with Endo-F2 or Endo-M could not remove the tetra-antennary N-glycans, either. These results are consistent with their reported substrate selectivity, that none of the above endoglycosidases is able to hydrolyze tetra- or higher branched N-glycans. To produce a glycosylated EPO precursor that is cleavable by existing endoglycosidases, we then expressed EPO with high mannose glycan from the HEK293T cell in the presence of mannosidase inhibitor kifunensine.34 As shown in Figure 1A, MALDI-TOF analysis indicated major glycans in this glycoform are Man9GlcNAc2 (M9), M8, and M7 (Figure 1A). This glycoform was processed by Endo-H, resulting in the GlcNAc-EPO acceptor (Figure S1, Supporting Information). Treatment of the complex type EPO with Endo-F3 led to no measurable cleavage of N-glycans from the recombinant EPO, as assessed by SDS-PAGE analysis (data not shown). The treatment of the recombinant EPO with Endo-F2 or Endo-M could not remove the tetra-antennary N-glycans, either. These results are consistent with their reported substrate selectivity, that none of the above endoglycosidases is able to hydrolyze tetra- or higher branched N-glycans. To produce a glycosylated EPO precursor that is cleavable by existing endoglycosidases, we then expressed EPO with high mannose glycan from the HEK293T cell in the presence of mannosidase inhibitor kifunensine.34 As shown in Figure 1A, MALDI-TOF analysis indicated major glycans in this glycoform are Man9GlcNAc2 (M9), M8, and M7 (Figure 1A). This glycoform was processed by Endo-H, resulting in the GlcNAc-EPO acceptor (2) that carries three GlcNAc moieties at the 3 N-glycosylation sites. LC-MS analysis confirmed the successful conversion (measured 19 969 Da, calculated 19 972 Da, as shown in Figure 1B). Subsequently, we attempted to remodel EPO to complex glycans by using EndoM-N175A mutant, which is known to be able to transfer complex type N-glycan to GlcNAc-protein.16 We selected the sialylated biantennary complex type N-glycan oxazoline (S2G2-Oxa) as the donor substrate to transfer it to the GlcNAc-EPO (EPO-Gn) acceptor (Scheme 1). As shown in Figure 1C, even with the addition of large excess of S2G2-Oxa and high concentration of enzyme (0.3 μg/μL), only one of the three N-glycosylation sites in EPO was partially transferred (found, 21 969 Da; calculated, M = 21 973 Da). This result indicated that the EndoM-N175 mutant was not efficient at transferring complex type glycan oxazoline to EPO with multiple N-glycosylation sites. The site of transglycosylation in product 3 was not further characterized due to the low efficiency of transglycosylation and the difficulty in isolating the product from the mixtures. In our previous test, the Endo-M mutant could not transfer complex type oxazoline to IgG either,35 although it was able to glycosylate GlcNAc-ribonuclease B.16 We also found that the EndoF3-D165A mutant, a glycosynthase derived from Endo-F3,22 was unable to glycosylate the GlcNAc-EPO either (data not shown). This is anticipated, as we have previously shown that the EndoF3-D165A mutant was specific for the core-fucosylated GlcNAc-protein acceptor and had only marginal activity on the nonfucosylated GlcNAc acceptor.22

**Generation of Core-Fucosylated EPO Glycoform Suitable for EndoF3 Glycosynthase-Catalyzed Transglycosylation.** Having confirmed that the nonfucosylated acceptor GlcNAc-EPO (2) could not be effectively transferred with an existing glycosynthase, we next sought to generate core-fucosylated EPO glycoform, Fucα1,6GlcNAc-EPO (EPO-GnF, S), which might serve as a good acceptor substrate for EndoF3 glycosynthase-catalyzed transglycosylation. Recently, we observed unexpected high core-fucosylation of Man5GlcNAc2 (M5) glycan (more than 50%) of EPO produced from a HEK293S GnTI knockout cell line.33 To characterize the underlying mechanism and to increase the fucosylation level of EPO-M5 as a potential starting material for glycan remodeling, we have generated a 293S GnTI−FUT8↑ cell line by lentiviral-mediated gene transfer and found that expression of EPO from this cell line yielded almost 100% homogeneous MSF glycan.33 This successful generation of this unusual glycoform prompted us to explore it to obtain the Fucα1,6GlcNAc-EPO as the key precursor for EPO glycan remodeling. For this purpose, we first tested recombinant Endo-H and Endo-A for deglycosylation of this EPO intermediate (Scheme 2). As shown in Figure 2A, Endo-H could effectively cleave three MSF’s on EPO,
generating EPO-GnF (5). LC-MS analysis confirmed the complete deglycosylation (deconvoluted, 20 406 Da; calculated, $M = 20\ 410\ Da$, Figure 2B; $m/z$ profile, Figure S2A). We found that Endo-A could also efficiently process EPO-M5F (4) to give the homogeneous EPO-GnF intermediate (5; data not shown).

**Glycan Remodeling of EPO with Homogeneous Sialylated Complex Type Glycan and Azide-Tagged Glycan.** Having successfully acquired the EPO-GnF (5) acceptor, we tested the transfer of S2G2-oxa by the EndoF3-D165A glycosynthase (Scheme 2). Incubation of EPO-GnF (5) with 60 mol equiv of the glycan oxazoline (20 equiv for each glycosylation site) and a 0.05 μg/μL concentration of glycosynthase for 30 min gave a single product (6) that appeared as a band about 4 kDa (equivalent to size of two S2G2 glycans) larger than that of the starting material on SDS-PAGE (Figure 3A). Meanwhile, the band of starting material EPO-GnF totally disappeared, indicating full conversion. LC-MS analysis showed that the transferred product (6) had a deconvoluted molecular weight of 24 411 Da (Figure 3B; $m/z$ profile in Figure S2B, Supporting Information), which corresponds to an addition of two S2G2 glycans (calculated MW 24 413). The transfer did not proceed in such a condition for another 30 min (Figure 3A, lane 3). In this case, a high regio-selectivity was achieved, and the glycan-remodeled EPO glycoform (6), which carries two sialylated N-glycans selectively at the N38 and N83 sites (as determined by tandem MS analysis below) and a His tag at the C-terminus, was readily isolated by Ni-NTA affinity chromatography and its purity was confirmed by ESI-MS analysis (Figure 3B). When more oxazoline (totally 180 ea) and enzyme (final concentration 0.2 μg/μL) were added and the incubation time was extended, a second band that was ca. 6 kDa larger than that of the starting material (EPO-GnF) appeared on SDS-PAGE (Figure 3C, lane 4). LC-MS confirmed that the second band was the transglycosylation product (7) that carries three sialylated S2G2 N-glycans (found, 26 414 Da; calculated $M = 26416\ Da$, Figure 3D). The transglycosylation result illustrated that, among the three glycosylation sites of EPO, two sites appear much more accessible to the glycosynthase than the third site. With extended reaction (“push” condition), the transfer of S2G2-oxa could reach approximately 60% on the third site (100% on other two other sites). The high efficiency of transfer is in sharp contrast to EndoM-N175A catalyzed transglycosylation with GlcNAc-EPO as the acceptor.

In addition to sialylated glycan, we also tested if EndoF3-D165A could transfer azide-tagged Man3GlcNAc2 (M3N3) glycan to an EPO-GnF acceptor (5; Scheme 2). As shown in Figure 3D, a major peak of 21 885 Da (8), representing transfer of two M3N3 glycans (calculated 21890 Da), appeared after the transfer reaction in LC-MS analysis. This site-selective transfer is very similar to the transfer reaction of sialylated glycan. The EPO-(M3N3)$_2$ glycoform could be further modified to add

![Figure 2.](image2.png)

Figure 2. Generation of the EPO-GnF acceptor. (A) SDS-PAGE analysis. Lane 1, EPO-M5F; lane 2, EPO-GnF. (B) LC-MS analysis of EPO-GnF.

![Figure 3.](image3.png)

Figure 3. Efficient remodeling of EPO to a homogeneous complex glycoform or an azide-tag glycoform with EPO-GnF as a starting material. (A) SDS-PAGE analysis of EndoF3-D165A catalyzed transglycosylation of sialylated complex type glycan. Lane 1, EPO-GnF; lane 2, EPO-GnF transferred with S2G2-Oxa in 30 min; lane 3, transglycosylation at 1 h; lane 4, transglycosylation overnight. (B) LC-MS analysis of EPO-(S2G2)$_2$. (C) LC-MS analysis of EPO-(S2G2)$_2$-3. (D) LC-MS analysis of EPO-(M3N3)$_2$. The major peak of 21 885 is EPO transferred with two M3N3 glycans. The minor peak of 21 145 is the product of transfer with one M3N3 glycan, while 22 625 Da is transferred with three M3N3 glycans.
polysialylated oligosaccharides to extend the serum half-life of EPO by click chemistry. A functional group could also be introduced to help study the activity of EPO.

**Determination of the Transglycosylation Site Selectivity in the Product (6) by LC-MS Analysis.** The interesting site selectivity in the EndoF3-D165A glycosynthase catalyzed transglycosylation of the EPO-GnF (5) that carries three Fucα1,6GlcNAc moieties prompted us to perform a detailed MS/MS of the transglycosylation product (6) to define the transferred sites. After digestion of 6 with endoproteinase Glu-C and trypsin, the glycopeptides generated were enriched via hydrophilic interaction on Amide 80 HILIC resin and subjected to MS/MS analysis. Representative spectra from glycopeptides containing glycosylation sites N38 (A) and N83 (B) obtained from digestion of the glycan-remodeled EPO (6) are shown in Figure 4.

The green peak in each spectrum was the intact glycopeptide ion signal. The peptide sequence was determined by a series of b and y ions, which are indicated by blue and red lines on the left part of the spectra. The oxonium ions at m/z 204.09, 366.14, 209.10, and 657.24 indicate the presence of HexNAc, Hex-HexNAc, NeuAc, and HexNAc-Hex-NeuAc, respectively. They were used as diagnostic markers to assess whether the predicted glycoform composition was correct. The fragment ions on the right half of the spectra were the glycosidic bond cleavage products. They were used to derive the sequence and composition of the glycan moiety as shown in Figure 4. For the glycopeptide with N38, the ion at m/z 1090.56 was the peptide NITVPDTK plus the most aglycone GlcNAc residue. It was used to verify peptide identity. The ion at m/z 1236.62 was the peptide bearing the GlcNAc+Fuc structure, indicating the presence of a core fucose moiety. We also annotated the inferred glycan sequences for some fragment ions in the figure.

For the glycopeptide with N83, the peptide plus the GlcNAc residue was at m/z 2562.32, and a series of glycosidic fragments were annotated in the spectrum. No glycopeptide containing...
N24 site was detected, which suggested that this site was not transglycosylated and thus was not trapped by the Amide 80 HILIC resin. Taken together, the LC-MS analysis demonstrated that among the three sites of EPO, N38 and N83 were the preferred sites for transglycosylation by Endo-F3 glycosynthase, while the N24 site was much less reactive.

**Site-Selective Glycan Remodeling of EPO.** The discovery of the selectivity in transglycosylation reactivity of the three glycosylation sites of EPO raised an exciting possibility to perform site-selective transglycosylation of this protein, transferring one glycan to two sites (N38 and N83), and adding a different glycan to the third site (N24). We designed the reaction strategy as follows: (1) transferring S2G2-oxa to site N38 and N83 by EndoF3-N165A in a controlled reaction condition, resulting in glycoform 6, (2) removing EndoF3-N165A and unreacted S2G2-oxa from the reaction mixture, (3) defucosylation of glycoform 6, and (4) adding the second Man3GlcNAc-azide oxazoline by Endo-A glycosynthase (Scheme 3). Successful implementation of this strategy led to the synthesis of glycoform 9 that carries two sialylated N-glycans at N38 and N83 sites and an azide tagged Man3GlcNAc2 glycan at the N24 site (Figure 5). Briefly, glycoform 6 was prepared by controlled transglycosylation of 5, giving the glycoform (6) that carries two sialylated N-glycans at N38 and N83 sites. To introduce an azide-modified Man3GlcNAc glycan using Endo-A as the enzyme, which could not use core-fucosylated GlcNAc-protein as the acceptor substrate, the fucose residue in the Fucor1,6GlcNAc at the N24 site of 6 was removed by treatment with the α1,6 fucosidase from *Lactobacillus casei*. It was found that this α-fucosidase was able to remove the fucose residue in the disaccharide Fucor1,6GlcNAc moiety (at N24), but not on the core fucose in the context of full length N-glycans (at the N38 and N83 sites), probably due to steric hindrance. A similar phenomenon with this fucosidase was observed in the glycosylated and deglycosylated Fc domain of IgG antibodies including rituximab (unpublished data). A recent report also showed that the α-fucosidase from *Bacteroides fragilis* had similar selectivity in defucosylation. Finally, Endo-A catalyzed transglycosylation of glycoform 9 with the azide-Man3GlcNAc oxazoline gave the novel glycoform 10. In the present case, we used an immobilized EndoF3-D165A for the first step transglycosylation in order to facilitate the removal of EndoF3-D126A from the reaction system. The glycosynthase was immobilized to an activated agarose bead containing an aldehyde group through reductive amination.

As shown in Figure 5A, the use of the immobilized glycosynthase led to a slight reduced yield of transglycosylation, giving EPO-(S2G2)$_2$ (6) as the major product, together with some unreacted EPO-GnF (5), partially transferred EPO-S2G2, and fully transferred EPO-(S2G2)$_2$ (7) as the minor byproducts. Selective defucosylation at the N24 site with the α-fucosidase gave glycoform 9 as the major product (major peak boxed in red in Figure 5B; found, 24,265 Da; calculated, M = 24,266 Da). The minor peaks are EPO-S2G2 with fucoses on two GnF moieties removed (22,116 Da) and intact glycoform 7 (26,414 Da). The final transfer was performed by the addition of EndoA and azide-Man3GlcNAc (M$_3$N$_3$) oxazoline. As illustrated in Figure 5C, a new major peak (boxed in red) was shown, which was the expected final product EPO-(S2G2)$_2$-M$_3$N$_3$ (glycoform 10, found, 25,005 Da; calculated, M = 25,005 Da). The glycan transfer reached approximately 50% after 2 h of reaction. A minor peak of 23,595 Da was the defucosylated EPO-S2G2 transferred with two M$_3$N$_3$’s. The results suggest that it is possible to perform site-selective glycan remodeling among the three N-glycans in EPO. In this case, the last enzymatic transglycosylation was not optimized, and the reaction was incomplete. The EPO was readily separated from the glycans and enzyme via the Ni-NTA affinity chromatography. However, it was still a mixture of the precursor 6 and the glycan remodeled final product, EPO-(S2G2)$_2$-M$_3$N$_3$ (10). While we did not attempt to purify the final product from the mixtures, the purification could be achieved through lectin-affinity chromatography, such as the blooddrop lectin that can selectively bind the Man$_n$GlcnAc$_m$ moiety. In practice, it would be ideal to push the reaction to completion so that a tedious separation of glycoform mixtures would be avoided. The introduction of an azide-modified N-glycan at a selective site will allow further selective modifications such as introduction of a fluorescent probe or another tag for functional studies.

The site-selectivity of EPO transglycosylation might be due to the difference in steric hindrance at different glycosylation sites. N38 and N83 sites of EPO seem more exposed than N24 sites, permitting selective introduction of two N-glycans at these two sites, and a different N-glycan at the N24 site. Previous site-specific glycosylation profiling of recombinant EPO indicated that the glycans attached at the N38 and N83 sites were mainly tetra-antennary glycans while that at the N24 site was a mixture of bi-, tri-, and tetra-antennary glycans. The data also imply that the N38 and N83 sites might be more accessible for enzyme processing to give the fully processed and highly branched tetra-antennary N-glycans as the major glycoforms, while the N24 site might be less accessible for enzymatic processing to yield the less branched glycoforms. These results appear to be consistent with our observation of site selectivity in the enzymatic glycan remodeling of EPO. As to the glycan remodeled EPO described in this work, the two sialylated N-glycans introduced at N38 and N83 could help to sustain the solubility and stability of the glycoprotein, while the azide modified N-glycan attached at the N24 site could be used to introduce other functional groups via click chemistry, such as a fluorescent tag or other chemical probe for functional studies.

**Figure 5.** Site-selective glycan remodeling of EPO. (A) LC-MS analysis of EPO-(S2G2)$_2$. (B) LC-MS analysis of defucosylated EPO-(S2G2)$_2$. (C) LC-MS analysis of EPO-(S2G2)$_2$-M$_3$N$_3$. DOI: 10.1021/acs.chembio.6b00382
studies of EPO. While the generality of this approach remains to be tested with additional multiply glycosylated proteins, the present discovery suggests that a combination of the distinct accessibility of glycosylation sites, the difference in substrate specificity of endoglycosidases, and the nature of the N-glycans could achieve site-selective modification of N-glycans in the context of a complex glycoprotein.

**Conclusion.** A chemoenzymatic glycan remodeling of erythropoietin (EPO) that carries three N-glycans is described. The combination of mammalian cell engineering and in vitro chemoenzymatic glycan remodeling represents a novel platform for producing homogeneous glycoforms of erythropoietin. An important finding from the present study is the discovery of highly site-selective transglycosylation of the three N-glycosylation sites in EPO, enabling selective introduction of different N-glycans in the glycoprotein. The present study represents the first example of site-selective glycan modification of a therapeutic protein carrying multiple N-glycans, which remains a challenging task and deserves further explorations.

**MATERIALS AND METHODS**

**Cell Culture.** Human HEK293T cells were cultured in a suspension in FreeStyle F17 serum-free medium supplemented with GlutaMAX (Life Technologies), incubating at 37 °C in 8% CO₂ and shaking of 140 rpm/min. The cells were passaged every 3–4 days with the initial seeding density of 3 × 10⁵ cells per mL. The culture of HEK293S GnTTFTUT8 followed the procedures previously reported by Yang et al.15

**Expression and Purification of EPO-HM and EPO-MSF (4) from Human Cell Line.** The high mannose type EPO (EPO-HM) was produced in the HEK293T cells by transient transfection in the presence of 5 μg/mL of kifunensine (Cayman Chemical, Ann Arbor, Michigan), a mannosidase inhibitor. The EPO-MSF (4) was produced in HEK293S GnTTFTUT8 cells. The construction of the EPO-S126V expressing plasmid, the transfection, and the purification procedures were described as by Yang et al.15

**Generation of EPO-Gn or EPO-GnF Acceptor.** The EPO-Gn acceptor was created by treatment of EPO-HM with Endo-Hf (New England Biolabs). A total of 200 μg of EPO-HM was treated with 1000 u of Endo-Hf in 100 μL of PBS buffer, at pH 7.4. The mixture was incubated at 37 °C for 1 h. The EPO-Gn acceptor was generated in a similar manner. A total of 400 μg of EPO-GnF was processed by 2000 u of Endo-Hf in 200 μL of PBS buffer.

**Synthesis of Oxaazolines.** Biantennary sialylated complex glycans oxazoline (S2G2-Oxa) was synthesized according to the previously reported protocol.10 Azido tagged Man3GlcNAc2 oxazoline (M3N₃-Oxa) was prepared according to the published procedures.36

**Glycan remodeling of EPO to Homogeneous Complex Glycoform.** In the initial trial, the GlcNAc-EPO was transfected with a biantennary sialylated complex glycan oxazoline (S2G2-Oxa) by Endo-M-N175A glycosynthase. A total of 100 μg of GlcNAc-EPO is mixed with 15 μg of Endo-M-N175A and 2 mg of S2G2-Oxa, in 50 μL of PBS buffer, at pH 7.4. The reaction mixture was incubated at 30 °C overnight. Aliquots were taken at different time points to monitor the reaction with either SDS-PAGE or LC-MS. The EndoM mutant catalyzed transfer turned out to be quite inefficient. Next, we tried to transfer S2G2-Oxa to an EPO-GnF acceptor with EndoF3-D165A glycosynthase. The transfer was carried out under two different conditions: a controlled or “push” reaction. Under controlled reaction conditions, 200 μg of EPO-GnF was mixed with 1.18 mg of S2G2-Oxa (60 ea) and 5 μg of EndoF3-D165A glycosynthase (final concentration 0.05 μg/μL), in 100 μL of PBS buffer, at pH 7.4. The mixture was incubated at 30 °C for 15–30 min. Under such conditions, the transglycosylation product is EPO-(S2G2)₂. EPO transferred with two S2G2 glycans. Under the “push” conditions, 50 μg of EPO-GnF was mixed with 300 μg (60 ea) of S2G2-Oxa and 2.5 μg of EndoF3-D165A (final concentration 0.1 μg/μL), in 25 μL of PBS buffer, at pH 7.4. After 30 min of incubation at 37 °C, another aliquot of S2G2-Oxa (120 equiv) and 2.5 μg of EndoF3-D165A (final concentration 0.2 μg/μL) were added, and the reaction was extended for 2 h overnight. Under these conditions, the main transglycosylation product is EPO with three S2G2 glycans.

**Transfer of Azide-Tagged Glycan to Fucα1,6GlcNAc-EPO (EPO-GnF) Acceptor by EndoF3-D165A Glycosynthase.** A total of 50 μg of EPO-GnF was mixed with 114 μg of azide-tagged Man3ClcNAc oxazoline (M3N₁-Oxa: 60 ea), 2.5 μg of EndoF3-D165A glycosynthase (final concentration 0.1 μg/μL), in 25 μL of PBS buffer, at pH 7.4. The mixture was incubated at 30 °C for 30 min. Afterward, another aliquot of M3N₁-Oxa (60 μg) and 2.5 μg of EndoF3-D165A (final concentration 0.2 μg/μL) were added, and the reaction was extended for an additional 30 min.

**Site-Selective Glycan Remodeling of EPO.** In the initial step, the EPO-GnF acceptor was transferred with S2G2 glycan to N38 an N83 glycosylation sites. To facilitate its removal after the reaction, the EndoF3-D165S glycosynthase was immobilized to an AminoLink resin according to our previous report.41 The glycopeptides were enriched via solid phase extraction with TSKgel Amide 80 HILIC resin, at pH 7.4. The mixture was incubated at 37 °C for 60 min in a head-to-end rotator. After LC-MS confirmation of the formation of a major EPO-(S2G2)₂ product, the immobilized EndoF3 mutant was removed by gentle centrifugation (100 g × 2 min). An overnight dialysis with a Slide-A-Lyzer MINI Dialysis Device (ThermoFisher) at 4 °C was performed to remove unreacted oxazoline and change the buffer to PBS, at pH 7.4. In a second enzymatic reaction, defucosylation was performed by adding 1 μg of AlfC (α1,6-fucosidase) to 15 μL of EPO-(S2G2)₂, intermediate product and incubating at 37 °C for 12 h. The completion of defucosylation was confirmed by LC-MS. In the final step, the transfer of M3N₃-Oxa to the N24 glycosylation site was catalyzed by EndoA. The defucosylated EPO-(S2G2)₂ was mixed with 3 μg of EndoA (final concentration 0.2 μg/μL) and 100 μg of M3N₃-Oxa (180 μg), then incubated at 37 °C for 90 min. The reaction was pushed with adding another 180 μg of M3N₃-Oxa and incubating for 90 more minutes. The final transglycosylation product was analyzed with LC-MS.

**Mass Spectrometry Analysis of Intact EPO and Released N-glycan.** Intact EPO was analyzed with liquid chromatography electrospray mass spectrometry (LC-ESI-MS) on an ExactivePlus Orbitrap system (Thermo Scientific) with a C8 column (Poroshell 300SB-C8, 1.0 × 75 mm, 5 μm, Agilent) with a 5–90% acetonitrile linear gradient in 6 min. The raw data were deconvoluted with MagTran (AmpGen). The PNGase-F-released N-glycan was analyzed with an Autoflex III Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics). The release, cleanup, and analysis of glycan followed the previously reported procedures.

**LC-MS Analysis of the Glycopeptides from EPO-(S2G2)₂.** To identify two transferred glycosylation sites, EPO-(S2G2)₂ protein was digested with endoproteinase Glu-C and trypsin. Intact glycopeptides were enriched via solid phase extraction with TSKgel Amide 80 HILIC resin according to our previous report.31 The glycopeptides were injected onto a C18 column (HSS T3 nanoACQUITY column 75 μm i.d. × 100 mm, 1.8 μm particle, Waters Corporation), and the effluent was introduced into a Waters SYNAPT G2 HDMS system (Waters Corp. Milford, MA). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics). The instrument was set to perform MSE (MALDI-TOF) mass spectrometer (Bruker Daltonics).
are collected during the low energy MS scan, and then the collision energy is ramped linearly over a range of voltages in the dissociation stage to fragment the precursor ions. The data produced during the experiment are used to reconstruct MS/MS spectra without the bias associated with data-dependent ion scanning. The process can be optimized not only to fragment the glycan portion but also to generate abundant b and y ions from the peptide backbone. Therefore, the MS² spectra are rich in fragment ions from both glycoside and peptide moieties of the glycopeptides analyzed.\(^\text{42}\)

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00282.

Supplementary figures for ESI-MS profiles of recombinant and glycan-remodeled EPO (PDF)

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**Notes**

The authors declare no competing financial interest.

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