Thrombopoietin (TPO) is a cytokine that primarily stimulates megakaryocytepoiesis and thrombopoiesis. TPO has a unique C-terminal tail peptide of about 160 amino acids that consists mostly of hydrophilic residues and contains six N-linked sugar chains. In order to investigate the biological function of the C-terminal domain, two series of mutations were performed. One is systematic truncation from the C terminus. Another is elimination of N-glycosylation sites in the C-terminal domain by Asn→Gln mutations. After the mutant proteins were expressed by mammalian cells, it was found that the elimination of the N-linked sugar sites did not affect the biological activity, whereas truncation of the C-terminal domain resulted in elevation of in vitro activity up to 4-fold. The C-terminal peptide itself was found to inhibit the in vitro activity. Moreover, both the C-terminal truncation and the elimination of the N-glycosylation sites decreased the secretion level progressively down to 1/10 that of wild type, and the amount of the mutant left in the cell increased. The N-glycosylation in the C-terminal region was found to be important for secretion of TPO. Among six N-glycosylation sites in the C-terminal region, two locations, Asn-213 and Asn-234, were found to be critical for secretion, and two other locations, Asn-319 and Asn-327, did not affect the secretion.

Thrombopoietin (TPO) is a hematopoietic cytokine that primarily regulates megakaryocytepoiesis and platelet production. Recently, TPO has been cloned independently by several groups from different species and identified as a ligand of the Mpl receptor (1–10). Human TPO (hTPO) consists of 332 amino acids and is divided into N- and C-terminal domains. The amino acid sequence of the N-terminal domain has about 23% identity to that of erythropoietin. This domain contains two disulfide bridges, located between Cys-7 and Cys-151 and between Cys-29 and Cys-85, which are essential for displaying full biological activity (11, 12). Truncated versions of TPO containing the N-terminal region, such as purified molecules from plasma or recombinant molecules, are known to be biologically active (1, 11, 13). The C-terminal region has a character that is quite distinct from other excreted growth factors. This domain contains six N-linked sugar sites and a large number of prolines, serines, and threonines. Some membrane-anchored growth factors, such as macrophage colony-stimulating factor (M-CSF), c-kit ligand, tumor necrosis factor-α, and epidermal growth factor family, have been known to contain membrane anchoring regions (14). Truncation mutagenesis of this region in M-CSF did not affect in vitro activity (15). However, the C-terminal domain of TPO does not have a hydrophobic transmembrane region like that of M-CSF. What is the biological function of this unique C-terminal domain? It was speculated that the enhancement of the circulating half-life might be one function of this domain, as in the case of EPO (10). More than this, at least two possibilities can be considered for the function of the C-terminal tail peptide. The first possibility is that some part of the sequence in the C-terminal domain contains a peptide region that is important for TPO activity. Another possibility is that the C-terminal domain is important for the secretion of TPO. To test these hypotheses, two series of mutation experiments were performed. One is the systematic truncation of the C-terminal domain. Another is the elimination of N-glycosylation sites in the C-terminal domain by replacing glycosylated asparagines with glutamines. The effect of these mutations on the secretion and function of TPO was investigated. It was found that the C-terminal domain plays an important role in the secretion of TPO.

EXPERIMENTAL PROCEDURES

Construction of C-Terminal Truncation or N-Glycosylation-defective Mutants of hTPO—The expression plasmid of wild type TPO, pHTF1, which included the full-length (amino acids 1–332) hTPO cDNA has been described previously (8). The construction of truncated genes was carried out by polymerase chain reaction (PCR). N-Glycosylation sites were eliminated by site-directed mutagenesis (Transformer site-directed mutagenesis kit, CLONTECH Laboratories, Inc.). All mutant genes were sequenced with an Applied Biosystems 373A nucleotide sequencer.

Expression of TPO Mutants—The expression vectors were transfected into COS cells by the DEAE-dextran method as described previously (8, 16). COS7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Conditioned medium was collected after 5 days of growth.

TaqMan Real-time Quantitative Reverse Transcription Polymerase Chain Reaction—TaqMan real-time quantitative PCR assay was performed according to the literature (17), with a few modifications. Total
RNA was recovered from COS7 cells using Isogen (Nippon Gene, Tokyo) and subjected to reverse transcription, quantitative PCR using TaqMan Gold reverse transcription PCR kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. About 2 ng of total RNA was converted to cDNA using 1.25 units of multiscribe reverse transcriptase. The target PCR primers T-3 (forward, 5′-TGC TTC GTG ACT CCC ATG TC-3′) and T-6 (reverse, 5′-CCC AGA ATG TCC TGT GCC TT-3′), as well as the TaqMan probe, were added to the cDNA solution and transferred to a thermocycler tube. The TaqMan probe (5′-FAM-TTC ACA GCA GAC TGA GCC AGT GCC C-TAMRA-3′, PE Applied Biosystems) contains the reporter dyes FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) on the 5′- and 3′-nucleotides, respectively. Target and internal control (TaqMan ribosomal RNA control reagents, Applied Biosystems) were reverse-transcribed at 50 °C for 2 min, 95 °C for 10 min, followed by up to 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min, and the amplified products were monitored by using a sequence detector model 7700 (Applied Biosystems).

Electrophoresis and Western Blotting—The TPO mutant protein in the supernatant from COS7 cells was purified by immunoprecipitation with rabbit anti-hTPO polyclonal antibody (18) and separated by SDS-polyacrylamide gel electrophoresis with Multigel 10/20 or 15/25 (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) as described by Laemmli (19). To detect the mutant TPO remaining in the COS7 cells, total cellular protein was also separated by SDS-polyacrylamide gel electrophoresis. In both cases, mutant TPO was detected by Western blot analysis. The protein was transferred to polyvinylidene difluoride membrane (Applied Biosystems Inc.) and incubated with biotinylated anti-TPO polyclonal antibody and then with streptavidine conjugated with alkaline phosphatase (Roche Molecular Biochemicals). TPO bands were visualized by developing with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

Determination of TPO Concentration—Concentration of mutant TPO in the supernatant of COS7 cells was determined by sandwich enzyme-linked immunosorbent assay (ELISA). In order to accurately determine the concentration of truncated mutants, two independent types of ELISAs were used: one with anti-hTPO polyclonal antibodies and another with two monoclonal antibodies that recognize different epitopes of hTPO.

Biological Activity of the Mutant hTPO—The in vitro biological activity of TPO mutants was determined with the TPO-dependent cell line FDCP-hMPL5 (20, 21). The cell line was established by transfecting human c-mpl cDNA into murine cell line FDC-P2, which is interleukin-3-, interleukin-4-, and GM-CSF-dependent. The assay procedure was as follows. FDCP-hMPL5 cells were washed three times with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells were plated in the 96-well tissue culture plate at a density of 5 × 104 cells/well, and a TPO standard or unpurified culture medium with secreted mutant was added. The plates were placed in a 5% CO2 incubator at 37 °C. After 3 days of incubation, the proliferation of the TPO mutant protein in the supernatant of COS7 cells was determined by sandwich enzyme-linked immunosorbent assay (ELISA). In order to accurately determine the concentration of truncated mutants, two independent types of ELISAs were used: one with anti-hTPO polyclonal antibodies and another with two monoclonal antibodies that recognize different epitopes of hTPO.

Biological Role of C-terminal Region of TPO

RESULTS

Design and Expression of Mutant hTPOs—The C-terminal domain contains six potential sites for N-linked glycosylation. They are at positions 176, 185, 213, 234, 319, and 327, as shown in Fig. 1. We chose to truncate the C terminus in steps of 20 amino acids. In this strategy, most of the N-linked sugar chains were deleted independently (Fig. 1a). The truncation was stopped at Cys-151 because the corresponding residue in human EPO, Cys-161, is required to display biological activity (22). In order to eliminate N-linked sugar chains of hTPO, glycosylated asparagines were replaced by glutamine (Fig. 1b). The mutations were introduced individually and in combination.

The wild type and mutant TPOs were expressed in COS7 cells with the DEAE-dextran method. All mutants were secreted from COS7 cells. Shifts in the molecular weight were observed according to their truncated length (Fig. 2). The secretion level of TPO mutants in COS cell was determined by Western blot analysis. The estimated concentrations of secreted mutants were summarized in Fig. 3. The secretion levels of the truncated mutants decreased as more of the C terminus was truncated (Fig. 3a), although the expression level of the three mutants, TPO311, TPO291, and TPO271, was almost the same as wild type (~35 nM). The secretion level decreased progressively down to 1/50 that of wild type TPO. The secretion level of TPO251 was about half that of wild type (22 nM). Finally, when 181 residues were removed, the secretion level of TPO151 decreased down to less than 5 nM. Contrary to what would be expected if the mRNA level (shown in Fig. 5) caused the decrease in expression, preliminary analysis indicated that truncation of the C-terminal region tends to result in an increase in mRNA level. The expression level, therefore, seems to depend on the length of the C-terminal region containing six N-linked glycosylation sites.

How does the N-glycosylation itself affect the secretion level of TPO? The secretion levels of the glycosylation site elimination mutants were investigated, and the results are summarized in Fig. 3B. The secretion level decreased progressively with the elimination of N-linked sugar chains. The secretion

![Fig. 1. Schematic drawings of the hTPO mutants. The four conserved cysteine residues are indicated by C, and the six N-glycosylation sites are indicated by closed triangles. a, hTPO truncated mutants. The terms at the left of the bars represent the mutant names, which uses the last amino acid number of each mutant. b, glycosylation site elimination mutants of hTPO. Mutant names are shown at the left of the bars.](http://www.jbc.org/Downloaded from http://www.jbc.org/2018/24)
level of the wild type, TPOQ_1, and TPOQ_56 mutants were approximately 40 nM. The secretion level of the mutants TPOQ_2, TPOQ_3, and TPOQ_4, in which one N-linked sugar chain was eliminated, were approximately 30 nM. Finally, the secretion level of the mutant lacking all N-linked sugar sites (TPOQ_123456) was only about 5 nM, which was almost 1/8 that of wild type. The secretion level of truncated mutant (TPO_1_5_1) was almost the same as that of the mutant lacking all N-glycosylation sites (TPOQ_123456), indicating that elimination of the glycosylation site is the major factor in decreasing expression in the truncation mutants. Preliminary mRNA analyses of the glycosylation elimination mutants (Fig. 3) indicated that elimination of N-glycosylation sites did not decrease the mRNA level, supporting the importance of glycosylation in secretion of TPO.

In order to complement the investigation of the secretion level of mutant TPO, the amount of unsecreted protein was investigated by Western blotting analysis. Fig. 4 shows the result of Western blotting analysis of the unsecreted TPO. Equal amounts of cell suspension were loaded in each lane. The amount of unsecreted TPO clearly increased with increasing truncation from the C terminus (Fig. 4A). Similar results were observed in the elimination of N-linked sugar chains (Fig. 4B). From the molecular size, it seems that there were no N-linked sugar chains attached to the unsecreted protein. Therefore, heterogeneity of the bands seems to be only from the O-linked glycosylation. The existence of the N-linked sugar chains of the C-terminal region is clearly important for the secretion of TPO.

In Vitro Activity of hTPO Mutants—The effect of the C-terminal region on the biological activity is of interest. The in vitro biological activity of both truncated and glycosylation site elimination mutants was measured by the FDCP-hMPL5 cell proliferation assay (21). The results are summarized in Table I. The activity of mutant TPOs was indicated by relative activity, with wild type set at 100%. The truncated mutants, except for
TPO showed biological activity in a dose-dependent manner. The truncated mutant TPO150 lacking Cys-151 was inactive. The specific activity of TPO mutants, calculated using the two independent ELISA systems, was found to be elevated with truncation of the C-terminal region. Full-length TPO displayed an ED50 value of 40 pM in the proliferation assay, whereas ED50 values for the mutants TPO171, TPO163, and TPO157 decreased to 12 pM, indicating that the short forms of TPO are more than three times more active than the wild type (TPO332).

The specific activity of glycosylation site elimination mutants revealed different features from the truncated mutants. As shown in Table I, all nine glycosylation site elimination mutants displayed almost the same in vitro biological activity as that of the wild type (TPO332), indicating that the existence of the N-linked sugar chain does not affect the biological activity. Because the biological activity of glycosylation site elimination TPO was approximately 1/4 of that of the TPO163, the C-terminal peptide region of TPO somehow inhibits the TPO activity.

### DISCUSSION

**Role of the C-Terminal Domain on the Biological Activity of TPO**—The in vitro biological activity of the truncated mutants showed an increase of up to 4-fold (see Table I). Two reasons can be considered for this activation. One is that N-linked glycosylation inhibits the receptor binding. Another possible reason is inhibition of activity by the C-terminal peptide itself. Previous studies showed that the removal of the N-linked sugar chains results in an enhancement of the in vitro biological activity of EPO (23) and GM-CSF (26). However, mutants lacking N-linked sugar sites have activity similar to that of the wild type (TPO332), suggesting that N-linked glycosylation does not affect the biological activity. Therefore, an increase in TPO activity is considered to be due to the removal of the peptide chain, possibly eliminating an unfavorable interaction with the TPO receptor (Mpl). The systematic truncation of the C-terminal peptide clearly results in progressively increasing biological activity. The biological activities and the secretion levels of mutants and the hydropathy of wild type TPO are plotted against the residue number in Fig. 5. It was found that the TPO mutants having more than 250 residues showed the same activity, which was about 1/4 of the maximum activity seen in the truncated TPO mutants, such as TPO157, TPO163, and TPO171. The interval between N-glycosylation sites at positions 234 and 319 contains an obvious hydrophobic region from residue 270 to 300 (see Fig. 5). Because the N-terminal functional domain of TPO has been proposed to be extremely hydrophobic (8), this hydrophobic region in C-terminal domain may associate with the N-terminal domain of TPO to inhibit TPO association with the receptor. It may be noted that there is a dibasic site (Arg-Arg) located at positions 244 and 245 and that the proteolysis at this dibasic site can remove the hydrophobic region in the C-terminal peptide (27).

The Role of the C-Terminal Domain on the Secretion of TPO—It is now clear that the C-terminal domain of TPO is important for effective secretion and that the N-glycosylation is the main factor in improving the secretion. A decrease in the secretion level after removing N-linked sugar chains was also reported in other glycosylated cytokines, such as EPO (23–25) and interferon-γ (28), in which the sugar chain is thought to reduce the susceptibility to proteases during secretion and to be involved in correct folding (29). However, the contribution of N-linked sugar chains to the secretion of TPO could not be identified. The glycosylated C-terminal region may act as a molecular chaperon to help folding and transportation of the N-terminal domain through organella.

Moreover, the C-terminal region seems to have a biochemically important character for effective glycosylation. The C-terminal region is very hydrophilic in comparison with the N-terminal domain (Fig. 5). The existence of many helix-breaking residues, such as proline and glycine, also suggests that this region is unstructured, which is supported by the results of secondary structure prediction, as well as the fact that the existence of C-terminal region does not contribute to the CD spectra (data not shown). Because N-linked glycosylation is usually found in flexible loop regions (30), the unstructured C-terminal region may allow efficient glycosylation.

The systematic truncation and elimination of the N-glycosylation sites did not affect the biological activity. The systematic truncation of the C-terminal peptide clearly results in progressively increasing biological activity. The biological activities and the secretion levels of mutants and the hydropathy of wild type TPO are plotted against the residue number in Fig. 5. It was found that the TPO mutants having more than 250 residues showed the same activity, which was about 1/4 of the maximum activity seen in the truncated TPO mutants, such as TPO157, TPO163, and TPO171. The interval between N-glycosylation sites at positions 234 and 319 contains an obvious hydrophobic region from residue 270 to 300 (see Fig. 5). Because the N-terminal functional domain of TPO has been proposed to be extremely hydrophobic (8), this hydrophobic region in C-terminal domain may associate with the N-terminal domain of TPO to inhibit TPO association with the receptor. It may be noted that there is a dibasic site (Arg-Arg) located at positions 244 and 245 and that the proteolysis at this dibasic site can remove the hydrophobic region in the C-terminal peptide (27).

![Residue Number](image)

**FIG. 5.** Summary of the secretion level and in vitro activity of truncated hTPO mutants. The in vitro activities (●) and secretion levels (▲) are plotted against the residue number, with the activity of TPO153 and secretion of TPO332 set at 100%, respectively. Hydrophathy was calculated using the program DNASIS (Hitachi Software Engineering), with window sizes of 10 residues (thin line) and 15 residues (thick line) according to Kyte and Doolittle (31). The positions of disulfide bridges (12) and the N-linked sugar chains of Groups I, II, and III are also shown.

![Mutant ED50](image)

| Mutant      | ED50 | Relative activity |
|-------------|------|------------------|
| WT (332)    | 38.7 | 100              |
| 311         | 30.0 | 129              |
| 291         | 27.2 | 142              |
| 271         | 32.6 | 118              |
| 251         | 26.6 | 146              |
| 231         | 18.6 | 208              |
| 211         | 19.6 | 197              |
| 191         | 16.2 | 238              |
| 171         | 11.8 | 326              |
| 155         | 11.7 | 329              |
| 157         | 11.7 | 331              |
| 151         | 18.1 | 214              |
| 150         | ND   | ND               |
| Glycosylation site elimination mutants | | |
| WT (332)    | 44.1 | 100              |
| Q1          | 36.9 | 129              |
| Q2          | 34.3 | 129              |
| Q3          | 44.1 | 100              |
| Q4          | 52.9 | 83               |
| Q56         | 41.6 | 106              |
| Q456        | 29.6 | 149              |
| Q3456       | 49.6 | 89               |
| Q23456      | 45.5 | 97               |
| Q123456     | 43.4 | 102              |

### Table I

| Mutant      | ED50 | Relative activity |
|-------------|------|------------------|
| WT (332)    | 38.7 | 100              |
| 311         | 30.0 | 129              |
| 291         | 27.2 | 142              |
| 271         | 32.6 | 118              |
| 251         | 26.6 | 146              |
| 231         | 18.6 | 208              |
| 211         | 19.6 | 197              |
| 191         | 16.2 | 238              |
| 171         | 11.8 | 326              |
| 155         | 11.7 | 329              |
| 157         | 11.7 | 331              |
| 151         | 18.1 | 214              |
| 150         | ND   | ND               |
| Glycosylation site elimination mutants | | |
| WT (332)    | 44.1 | 100              |
| Q1          | 36.9 | 129              |
| Q2          | 34.3 | 129              |
| Q3          | 44.1 | 100              |
| Q4          | 52.9 | 83               |
| Q56         | 41.6 | 106              |
| Q456        | 29.6 | 149              |
| Q3456       | 49.6 | 89               |
| Q23456      | 45.5 | 97               |
| Q123456     | 43.4 | 102              |

* ED50 values are the concentrations producing 50% of the maximum biological activity.
* Relative activities are expressed as the activity of the wild type (WT) equal to 100%.
* ND, not detected.
loration sites further clarified the most important N-glycosylation sites for TPO secretion. The six sites of N-linked glycosylation can be divided into three groups (Groups I–III) according to location and effect on secretion. Group I involves the N-linked glycosylation sites at 176 and 185, Group II involves the sites at 213 and 234, and the Group III involves the sites at 319 and 327 (see Fig. 5). Truncation or elimination of Group III still showed the same secretion level as the wild type, whereas the removal of the N-linked sugar chain of Group II caused a large decrease of the secretion level (Fig. 3). The N-linked glycosylation site of Group I showed only a slight decrease in secretion of mutant TPO. Therefore, the N-linked glycosylation belonging to Group II (positions 319 and 327) is not necessary for the effective secretion of TPO. It also indicates that the N-linked sugar chain of Group II caused a large decrease of the secretion level (Fig. 3). The N-glycosylation belonging to Group II is most important for the secretion of TPO. Because TPOs have been cloned from five species, human (1), murine (2), canine (5), rat (8, 9), and porcine (10), the N-glycosylation sites of their C termini can be compared. Although high sequence homology (83% sequence identity between human and porcine) is found in the N-terminal domain, there is less identity in the C-terminal domains (67% identity). There are six, 7, 8, 5, and 6 N-glycosylation sites in the C-terminal domain of TPO from human, murine, canine, rat, and porcine, respectively. The conserved sites for N-glycosylation among TPOs from these five species correspond to the first four sites of hTPO at positions 176, 185, 213, and 234 belonging to Group I and II in humans. The conservation of the N-glycosylation in these TPOs supports our finding that the Group II N-glycosylation sites are most important for secretion of TPO. The finding that the existence of the C-terminal region increases the secretion level of TPO may provide a useful approach to improving the secretion level of other proteins by adding a sugar-rich, “shuttle” peptide tail.

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Functional Analysis of the C-terminal Region of Recombinant Human Thrombopoietin: C-TERMINAL REGION OF THROMBOPOIETIN IS A "SHUTTLE" PEPTIDE TO HELP SECRETION

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