The Glycan Domain of Thrombopoietin Enhances Its Secretion†

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ABSTRACT: Thrombopoietin (TPO) is the major regulator of megakaryocyte development and platelet production. The hormone is structurally characterized by an amino-terminal receptor binding domain (amino acid residues 1−152) predicted to encode a left-handed four-helix bundle structure, and a carboxyl-terminal domain (residues 153−335) that is remarkable for its abundant carbohydrate modification and a lack of homology to other proteins. To investigate the functional role of the carboxyl-terminal glycan domain, we generated truncated forms of murine TPO (TPO1−238, TPO1−174, and TPO1−152) and glycomuteins in which the predicted asparagine (N)-linked sites of glycosylation were sequentially mutated to glutamine (Q), and assayed their secretion and function by comparing them to the native sequence (TPO1−335). Following transient transfection of the corresponding cDNA expression vectors into mammalian cell lines, the secretory efficiencies of the proteins were compared with those of the native hormone. Transfection efficiencies were monitored by cotransfection and reporter gene assay, and TPO secretion was assessed by functional and immunologic assays. We found that full-length TPO was 5−29-fold more efficiently secreted than any of the truncated forms of the hormone in fibroblast and hepatocyte cell lines. Elimination of carboxyl-terminal sites of N-linked glycosylation had a minor impact on secretion of the protein. We conclude that the carboxyl-terminal domain of TPO serves the important role of enhancing secretion of the protein, and in this manner functions as a prosequence.

Thrombopoietin (TPO)[1] is the principle cytokine which regulates megakaryocyte development and platelet production (*1*). TPO acts at both early (*2*) and late stages of megakaryopoiesis (*3*, *4*), alone and in synergy with other cytokines (*5*). The hormone also acts in synergy with erythropoietin (EPO) to stimulate erythropoiesis (*6*). Subsequent studies have revealed that TPO is both necessary and sufficient for full MK maturation (*6*). The biological effects of TPO are not limited to the MK lineage, as BFU-E, CFU-GM, and CFU-MK are expanded by TPO treatment in normal and myelosuppressed mice (*7*). Moreover, TPO affects the survival and proliferation of primitive hematopoietic stem cells in vitro (*8*, *9*) and in vivo (*10*). In this manner, clinical benefit may be derived from TPO's utility in enhancing peripheral blood stem cell collection (*11*), and in ex vivo expansion of primitive hematopoietic cells (*12*). As a therapeutic agent, TPO has been shown to speed platelet recovery following myelosuppressive therapy in mice (*13*, *14*), in non-human primates (*15*), and in cancer patients (*16*, *17*). It is expected that the protein will contribute greatly to patient care by enhancing platelet recovery following myelosuppressive therapy and in states of primary marrow failure.

Like EPO, growth hormone, and other members of the hematopoietic growth factor family, the amino-terminal region of TPO is predicted to fold into a left-handed four-helix bundle protein. The amino acids of this domain (residues 1−152) are remarkably homologous with those of EPO [22% identical, and an additional 24% that is similar (*18*)]. Many groups have demonstrated that the amino-terminal (or cytokine-like) domain of TPO is adequate for receptor binding, signaling, and proliferation (*19*, *20*). The receptor binding domain contains three sites of serine or threonine (O)-linked glycosylation, but is devoid of N-linked glycosylation (*21*).

Unlike all known members of the hematopoietic cytokine family, in addition to the four-helix bundle, the TPO gene encodes a carboxyl-terminal polypeptide extension (residues 153−335), a serine- and proline-rich domain that is remarkable for its abundant carbohydrate modification and a lack of homology to other known proteins. Glycosylation of this carboxyl-terminal (or glycan) domain has been experimentally elucidated (residues 153−246) and predicted (residues 246−332) in human TPO (*21*). The amino-terminal half of the glycan domain of human TPO contains four sites of N-linked glycosylation and five sites of O-linked glycosylation; the homologous region of murine TPO is predicted to encode five sites of N-linked glycosylation. The pattern of glycosylation in the remaining carboxyl-terminal region has not been experimentally determined; however, there are two additional consensus sequons for N-linked glycosylation, and

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[1] Abbreviations: TPO, thrombopoietin; EPO, erythropoietin; MK, megakaryocyte; BFU-E, burst-forming unit erythrocyte; CFU-GM, colony-forming unit granulocyte macrophage; CFU-MK, colony-forming unit megakaryocyte; $NaDodSO_4$, sodium dodecyl sulfate; TGF, transforming growth factor; AR, amphiregulin; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor.

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16 serine and threonine residues which potentially could serve as sites of O-linked glycosylation. The glycosylation of TPO accounts for approximately one-half of its observed 70 kDa molecular mass. Not surprisingly, the interspecies homology of TPO is greatest in the amino-terminal receptor binding domain; however, the carboxyl-terminal domains are 61% identical in the human and murine homologues, suggesting that it also serves an important physiologic function.

In contrast to the structurally and functionally well-characterized amino-terminal domain, little is known about the function of the carboxyl-terminal domain other than that it appears to prolong the circulatory half-life of the protein (*22*). On the basis of our preliminary observations that truncated TPO muteins were poorly produced, we postulated that the carboxyl-terminal domain serves to enhance secretion. To test our hypothesis, we compared the immunologic and biologic activity of several murine TPO mutations to that of the native sequence. We generated four murine TPO expression vectors encoding full-length (TPO1−335) or three truncated forms (TPO1−238, TPO1−174, and TPO1−152) and expressed them in multiple mammalian cell lines that are representative of the natural sources of the hormone. TPO1−174 is similar in size to a naturally occurring proteolytic product of the full-length protein (*23*). We found that truncation of the carboxyl-terminal domain of TPO yielded progressively diminishing levels of protein secretion. We demonstrated these differences in secretory efficiency in all three cell lines that were tested, and conclude that the glycan domain of TPO enhances secretion. In an effort to distinguish the effects of N-linked glycosylation from the contributions of the polypeptide per se, we generated glycomuteins, sequentially mutating the seven potential sites of N-linked glycosylation, and assessed the secretory efficiency and specific activity of these proteins. Mutation of glycosylation sites reduced the molecular mass of the protein but did not substantially diminish its level of secretion.

MATERIALS AND METHODS

PCR Mutagenesis. The cDNA encoding murine TPO (previously described in ref *18*) was used as a template for PCR-based mutagenesis. To facilitate iodination and purification, PCR-based site-directed mutagenesis was used to add a polytyrosine, polyhistidine (pYpH) terminus and to mutate an Arg-Arg (residues 153 and 154) potential cleavage site to Gln-Gln (QQ). Oligonucleotides were designed to generate four forms of murine TPO, the full-length form (TPO1−335) and three truncated forms (TPO1−238, TPO1−174, and TPO1−152), with a pYpH carboxyl terminus using a strategy we have previously described (*24*). The secretory efficiency (as measured with an ELISA) and function (as measured with the MTT assay; see below) of TPO1−335 did not differ from those of wild-type mTPO, confirming that mutation of the Arg-Arg cleavage site and addition of the pYpH tail were functionally silent. Site-directed mutagenesis was also used to substitute glutamine (Q) codons for one to seven asparagine (N) codons at residue positions 176, 188, 213, 228, 235, 315, and 330. Figure 1 illustrates the cDNA constructs utilized in this study. Each TPO construct (encoding native TPO, truncation, or mutation of N to Q) was cloned into the mammalian expression vector pDX (*25*).

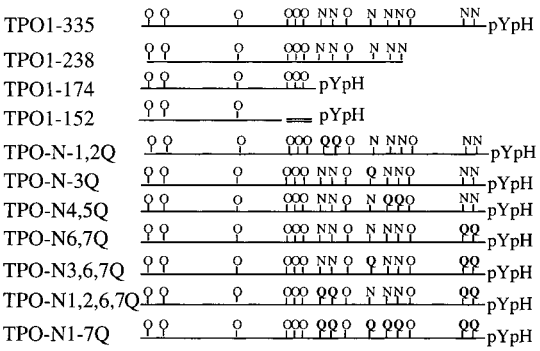

FIGURE 1: Diagram of TPO muteins, truncations, and glycomuteins. The four lengths of TPO cDNA and the glycomuteins used in our studies are illustrated. Each mutein contains receptor binding residues 1−152, and all but TPO1−238 have a polytyrosine, polyhistidine (pYpH) carboxyl terminus. Experimentally identified and predicted N-linked sites of glycosylation are denoted by N, and experimentally identified O-linked glycosylation sites are denoted by O; these sites have been identified by Hoffman and colleagues (*21*) in human TPO. TPO1−335 includes all glycosylation sites. TPO1−238 includes the experimentally identified (in human TPO) N-linked glycosylation sites, but does not include the two carboxyl-terminal predicted N-linked sites and several potential O-linked sites. TPO1−174 includes three O-linked sites of glycosylation without any of the N-linked glycosylation sites. TPO1−152 is identical in length to TPO1−174, but encodes only TPO residues 1−152 and additionally a linker region made up of alanine, glycine, and serine residues, without potential glycosylation sites. One mutant construct without the 22-amino acid "spacer" between the TPO residues and pYpH terminus exhibited substantially reduced activity, probably due to the proximity of pYpH to the active binding residues of the hormone. Glycomuteins were generated to mutate each N-linked glycosylation site to Q in single, paired, and grouped mutations. Glycomuteins are denoted by ordinal reference to the relative position of the site(s) of mutation of N to Q within the glycan domain. The template cDNA used for all constructs contains a mutation of the potential Arg-Arg cleavage site at residues 153 and 154 to Gln-Gln and a functionally silent substitution (Ala 279 to Thr). The cDNA sequence of each TPO glycomutein and truncation was verified by restriction mapping and DNA sequencing, and the cDNA was subcloned into a mammalian expression vector, pDX (*25*).

Transfection of Cell Lines. Purified full-length and mutein cDNA expression vectors were cotransfected using Lipofectamine (Life Technologies), Lipofectin (Life Technologies), or calcium phosphate into the rodent fibroblast cell lines BHK and CHO or the human hepatoma cell line HepG2, with a second plasmid encoding RSV-CAT (chloramphenicol acetyltransferase) at $^1/_{10}$ the concentration of the mutein cDNA (*26*), to control for transfection efficiency. Cells were then cultured in DMEM with 2% fetal calf serum with triple antibiotics. At 24−72 h post-transfection, supernatants were collected and cells counted and lysed. Supernatants were assayed for TPO (see below) and lysates for CAT activity (see below). To develop stable transfected cell lines, each TPO encoding cDNA was cotransfected with a plasmid encoding dihydrofolate resistance, at $^1/_{10}$ the concentration of the cytokine-containing plasmids. Cells were selected and grown in the methotrexate-containing medium, and then assayed for TPO activity to confirm the stable production of TPO.

Metabolic Labeling. In some experiments, cDNA expression vectors were used to produce metabolically labeled proteins. Six hours after transfection, cultures were trypsinized and split into two plates to allow metabolic labeling of half the transfected cells while the remaining cells were left

unlabeled in standard culture medium. Eighteen hours after transfection, adherent cells were washed and the supernatant was replaced with Met and Cys deficient medium (Gibco BRL) for 1 h. This medium was again replaced with Met and Cys deficient medium containing 1% dialyzed FCS, antibiotics, and 50 μCi/mL ^{35}S-labeled Met and Cys (NEN Life Science Products, Inc., NEG-072 EXPRE^{35}S^{35}S) and incubated overnight. Supernatants were then collected and clarified, and cells were trypsinized, counted, and lysed for CAT assays. The parallel nonlabeled culture supernatant was evaluated for mTPO with an ELISA (see below).

Immunoprecipitation. Immunoprecipitation of metabolically labeled tissue culture supernatants was performed using standard protocols. Two anti-peptide antibodies were generously provided by Kirin Pharmaceuticals. Each anti-peptide antibody is directed against a region of the receptor binding domain of rat TPO. RT1 is directed against a 20-residue region of the putative first helix (P^{9}RLLNKLLRDSYLLHR^{28}), and RT2 is directed against a 21-residue segment of the putative AB loop (F^{46}SLGEWKTQTEOSKAQDILGA^{66}). Murine and rat sequences are highly conserved in this region, differing at two and one residues, respectively (anti-peptide antibodies directed against similar regions of human TPO are described in ref *27*). Briefly, 1 mL of metabolically labeled tissue culture supernatant was precleared by incubation with 20 μL of protein A−agarose beads (Santa Cruz Biotechnology) for $^{1}/_{2}$ h; phenylmethanesulfonyl fluoride, leupeptin, and aprotinin were added to diminish the level of proteolysis, and 1% Tween 20 was used to diminish the level of nonspecific binding. Each supernatant was then incubated overnight with the anti-TPO peptide antibody (10 μg/mL) at 4 °C. Protein A−agarose beads were added to precipitate the antibody−TPO complex, and the beads were washed with RIPA buffers [10 mM Tris (pH 7.5), 1% Triton X-100, 1% deoxycholic acid, 0.1% $NaDodSO_4$, and 5 mM EDTA, initially with 0.7 M NaCl followed by the same buffer with 0.15 M NaCl], then denatured, and eluted from Staph A beads by boiling in a loading buffer with 2% β-mercaptoethanol. The immunoprecipitated TPO forms were size-fractionated by electrophoresis through 10% polyacrylamide gels, soaked in Amplify enhancer (Amersham Life Science), dried, and exposed to film with intensifying screens for 5−30 days.

Biological Activity Assay. Baf/3 cells transfected with the Mpl receptor were used to assay for TPO activity (*18*). Cells were grown and maintained in IL-3, washed, plated at a concentration of 10 000 cells per well in 100 μL of medium in 96-well plates, and incubated for 36 h with serial dilutions of tissue culture supernatants. Wild-type mTPO supernatant at a known concentration was used to generate a standard curve and determine maximal proliferation of Baf/Mpl cells for each assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma] was then added followed 5 h later by a lysis buffer. Optical density was measured using an ELISA plate reader (EL-340 Biotek Instruments Inc.) to measure the absorbance at 570−630 OD to assess the intracellular conversion of tetrazolium to formazan. Sample activity was determined by comparison to the standard at half-maximal proliferation.

Reporter Gene Assay. Standard protocols were used to assay CAT activity (*26*, *28*). Cellular lysates were diluted in 0.25 M Tris and incubated with acetyl CoA and [^{14}C]-chloramphenicol. Following ethyl acetate extraction, thin-layer chromatography was performed to allow quantitation of chloramphenicol acetylation. If the transfection efficiency differed significantly between samples (by more than 4-fold), we did not include the results from corresponding supernatants in our analysis.

Table 1: Relative Secretion of TPO Truncated Muteins[a]

	% secretion		
	IP/phosphorimage	ELISA	% activity
TPO1−335	100 ± 5.5	100 ± 3.9	100 ± 6.3
TPO1−335	18.7 ± 7.1	ND	26.9 ± 2.4
TPO1−174	12.5 ± 5.3	10.6 ± 2.7	48.4 ± 7.1
TPO1−152	3.4 ± 2.1	2.8 ± 0.8	20.6 ± 5.6

[a] Secretion of truncated TPO muteins, TPO1−238, TPO1−174, and TPO1−152, was compared with that of native TPO1−335, and assessed by three different methods, phosphorimage analysis of immunoprecipitated bands, ELISA, and activity. Secretion and activity values were normalized by reporter gene assays (to control for transfection efficiency) and by cell count, and then expressed as a percentage of TPO1−335 secretion. The quantity of immunoprecipitated ^{35}S-labeled protein was assessed by phosphorimaging (Molecular Dynamics PhosphorImager, using Imagequant), compared with that of [^{35}S]TPO1−335, and adjusted for the number of Met and Cys residues present in the two proteins to compare the secretion of each truncation mutein relative to that of native TPO. Each truncated protein was measured in parallel with TPO1−335 in two or three separate experiments; pooled results are shown with the standard error of the mean (SEM) of these measurements. The immunologic measurement of TPO1−238 was made only by quantitation of the ^{35}S-labeled supernatant. Additional assays of TPO1−174 and TPO1−152 were performed with a murine TPO ELISA using a purified murine TPO standard with a similar molecular mass, and they were compared with TPO1−335 in three to five additional experiments. The activity of each TPO form was measured in a proliferation assay using Baf/3 cells expressing the Mpl receptor to detect the activity of the transiently transfected protein in the supernatant.

Immune Assay. Supernatants were tested in paired samples using a commercial ELISA for mTPO (Quantikine M from R&D Systems). The manufacturer's supplied standard (70 kDa TPO) and two forms of purified mTPO (70 kDa and a mixture of 18, 22, and 30 kDa truncated mTPO forms kindly provided by Zymogenetics Inc.) were used to standardize the assay for TPO1−335 and for TPO1−174 and TPO1−152, respectively. The ELISA was 10-fold more sensitive to the full-length form of mTPO. Supernatants of glycomuteins which were as active as full-length TPO (as anticipated) in proliferation assays were also as readily detected by this commercial immune assay, suggesting that the antibodies used for detection are not directed against sites of N-linked glycosylation.

RESULTS

Secretion of mTPO1−335 and Truncated Forms. Secretion of the four forms of mTPO was evaluated with an assay of supernatants from transiently transfected BHK, CHO, and HepG2 cells. In separate cultures, plasmids encoding TPO1−335, TPO1−238, TPO1−174, and TPO1−152 were cotransfected with a plasmid encoding chloramphenicol acetyltransferase as described in Materials and Methods. The metabolically labeled supernatants were subject to immunoprecipitation (IP) using anti-TPO peptide antibodies directed

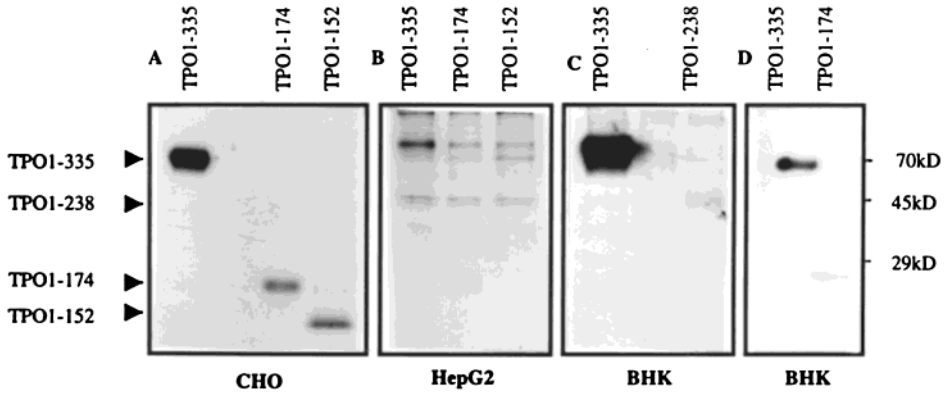

FIGURE 2: Secretion of TPO truncations. Shown are representative exposures of the immunoprecipitation of ^{35}S-labeled TPO from the supernatant of cells transiently transfected with mTPO cDNA forms; CHO cells (A), HepG2 cells (B), and BHK cells (C) were size-fractionated by polyacrylamide electrophoresis, dried, and exposed to film for 5−30 days (nontransfected HepG2 cells produce low levels of TPO). Panel A shows TPO1−335, 1−174, and 1−152. Panel B shows TPO1−335, 1−174, and 1−152. Panel C shows TPO1−335 and 1−238. Panel D shows TPO1−335 and 1−174. TPO1−152 in panel B cannot be detected by the eye or by a phosphorimage band significantly above background. However, its secretion is confirmed by activity in bioassays.

against a region of TPO present in all truncated forms utilized in our experiments. The quantitative results are shown in Table 1, and representative immunoprecipitation results from BHK, CHO, and HepG2 cells are shown in Figure 2. In each experiment, truncated TPO cDNAs were transfected in parallel with TPO1−335. When we corrected for cell number, CAT activity (a measure of transfection efficacy), and the relative number of Met and Cys residues present in each form of TPO, the relative level of secretion of the three truncated forms compared to that of TPO1−335 was 3.4−18.7%. In some experiments, TPO1−152 could not be detected by immunoprecipitation despite secretion assessed by detectable activity in the more sensitive MTT assay. ELISAs (using both native-length and truncated TPO standards) of unlabeled supernatants transfected on a smaller scale showed similar results, which are also shown in Table 1. The activity of supernatants from each transfection (or of parallel cultures of unlabeled supernatants, as described above) was also measured in proliferation assays using Baf/3 cells transfected with the Mpl receptor. As shown in Table 1, supernatants from cells transfected with TPO1−152 are 15−25% as active as TPO1−335. As both immunologic methods of detection indicated relatively lower levels of secretion of the truncated forms of TPO, the activity results suggest that the specific activity of the truncated forms is greater than that of TPO1−335.

Stability of TPO-Containing Supernatants. To determine whether the half-life of the protein in tissue culture may contribute to the apparent secretory efficiencies of TPO1−335 and TPO1−152, supernatants from established cell lines which secrete 5−10-fold more protein than transiently expressing cells were tested in serial bioassays. Each supernatant was incubated free of cells at 37 °C and tested on three consecutive days for biological activity using Baf/3 cells transfected with the Mpl receptor in MTT proliferation assays. Both TPO1−335 and TPO1−152 remained stable over the 3 day period of the experiment, as shown in Figure 3.

Secretion and Function of Glycomuteins. To distinguish the effects of glycosylation from the effects of the polypeptide sequence, we assessed the secretion of glycomuteins in which the N-linked sites in the glycan domain of TPO were mutated to Q. Glycomuteins were constructed at single and

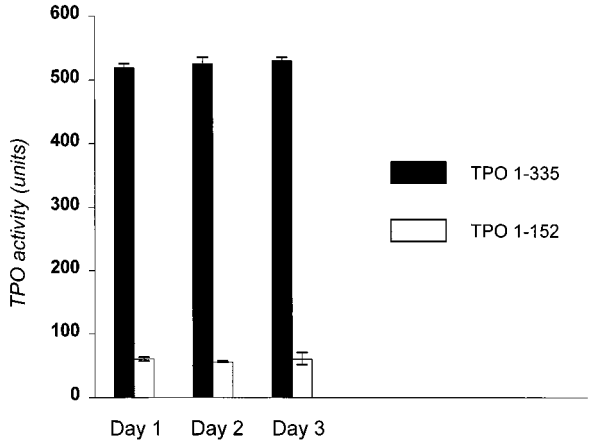

FIGURE 3: Stability of TPO1−335 and TPO1−152. Shown are activity measurements of TPO1−335 ($n = 2$) in black and TPO1−152 ($n = 3$) in white from supernatants from established cell lines secreting stable quantities of TPO. Activity assays were performed from supernatants stored at 4 °C, on day 1, and then from supernatants incubated free of cells at 37 °C on days 2 and 3 to test for the persistence of activity. Error bars define the standard error of the mean. Activity units are arbitrary; 25 units equals 30 ng of purified murine TPO.

multiple positions, encompassing all the predicted N-linked glycosylation sites, and then combined to test the cumulative effect of mutation at some or all sites of N-linked glycosylation (see Figure 1). N-Linked carbohydrate did not appear to influence the specific activity of TPO, as all but the most mutated of the glycomuteins exhibited a similar proliferative and immunologic activity (Table 2).

To assess whether the polypeptide or the N-linked carbohydrate of the glycan domain of TPO is responsible for the reduced secretory efficiency of the TPO truncation muteins, we measured the levels of protein production of the glycomuteins. Each cDNA was transiently transfected into BHK cells and compared to the native TPO expression vector in paired experiments, using ^{35}S metabolic labeling. Cotransfection of CAT reporter plasmids assured equivalent levels of transfection efficiency. There was little loss of secretory efficiency with mutation of paired or single sites of N-linked glycosylation (TPO−N1,2Q, TPO−N3Q, TPO−N4,5Q, or TPO−N6,7Q) as shown in panels A−C of Figure 4 and Table 1. Also of note in these experiments, while

Table 2: Relative Secretion of TPO Glycomuteins[a]

	% secretion	% activity
TPO1−335	100 ± 3.1	100 ± 6.3
TPO−N1,2Q	58 ± 13	67 ± 8.8
TPO−N3Q	106 ± 7.5	99 ± 11
TPO−N4,5Q	95 ± 6.3	98 ± 4.8
TPO−N6,7Q	73 ± 28	79 ± 9.4
TPO−N3,6,7Q	71 ± 17	72 ± 6.0
TPO−N1,2,6,7	53 ± 23	70 ± 8.1
TPO−N1−7Q	57 ± 17	18 ± 6.5

[a] Table 2 shows the levels of secretion of TPO glycomuteins relative to that of TPO1−335 from transiently transfected cells (BHK, CHO, and HepG2). Each glycomutein construct was tested in two to five separate experiments using at least two different plasmid preparations; the standard error of the mean of these measurements is shown. Supernatants were additionally assayed for activity in a proliferation assay using Baf/3 cells expressing the Mpl receptor. In parallel experiments, each plasmid was cotransfected with a reporter gene and secretion assessed by phosphorimaging of radiolabeled immunoprecipitated TPO forms, or by ELISA of unlabeled supernatants. Secretion and activity values were normalized by reporter gene assays (to control for transfection efficiency) and by cell count, and then expressed as a percentage of TPO1−335 secretion.

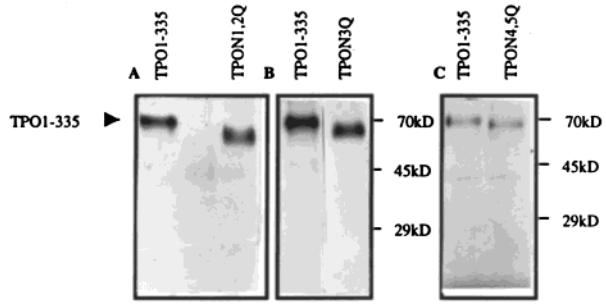

FIGURE 4: Secretion of TPO glycomuteins. Shown are representative exposures of immunoprecipitation of ^{35}S-labeled TPO glycomuteins from the supernatant of BHK cells transiently transfected with the glycomutein encoding mTPO cDNA, size-fractionated by polyacrylamide electrophoresis, dried, and exposed to film for 5 days. Each glycomutein is shown in comparison with the native form of the hormone, TPO1−335. Panel A shows TPO−N1,2Q. Panel B shows TPO−N3Q. Panel C shows TPO−N4,5Q. The quantity of immunoprecipitated ^{35}S-labeled protein was assessed by phosphorimaging (Molecular Dynamics PhosphorImager, using Imagequant), compared with [^{35}S]TPO1−335, and normalized for cell count and transfection efficiency. Glycomutein secretion in these experiments was not significantly different from that of TPO1−335 (see Table 1).

mutation of N176, N188, N213, N315, and N335 each resulted in a lower observed molecular mass following size fractionation by electrophoresis, concomitant mutation of N228 and N235 resulted in less size reduction than the other paired mutations, suggesting that only one of these potential sites is glycoslylated in BHK cells. The effects of the cumulative loss of N-linked glycosylation sites were also tested. TPO−N3,6,7Q, TPO−N1,2,6,7Q, and TPO−N1−7Q (see the legend of Figure 1 for descriptions) were transfected in parallel with TPO1−335. Cell counts and reporter gene assay results were used to normalize the effects of cell number and transfection efficiency. On the basis of ELISA and metabolic labeling measurements (Table 2), TPO−N3,6,7Q and TPO−N1,2,6,7Q, like TPO−N1,2Q, TPO−N3Q, and TPO−N6,7Q, were secreted as well as TPO1−335. However, TPO−N1−7Q and TPO−N1,2,6,7Q were secreted at a level that is 50% of that of TPO1−335 and the other single and composite glycomuteins.

DISCUSSION

Several groups have reported that truncated human TPO, encoding only the receptor binding domain of the hormone, functions normally in vitro (*19*, *29*). We have found similar results for the murine protein. It is not surprising that the region homologous to other cytokines in the hematopoietic growth factor family, alone, is responsible for biologic activity. However, other than the enhancement of protein survival in the circulation (*22*, *30*), the function of the unique glycan domain has not been well studied. In this paper [as we reported in abstract form (*31*)], we have demonstrated that truncation results in the progressive loss of secretory efficiency, suggesting a second role for the glycan domain of TPO, enhancing secretion of the protein. Of note, Ahn and colleagues (*32*) have reported an absence of secretion of truncated human TPO forms from insect cells, from which the native protein was secreted. We have also observed (Table 1) that carboxyl-terminal truncated TPO forms appear to be more potent than when the glycan domain is intact. This apparent increase in specific activity with truncation has been observed by others with human TPO (*23*, *30*) and murine TPO (T. Kato, personal communication, October 1999), suggesting the glycan domain of TPO also serves to inhibit activity of the receptor binding domain.

Enhanced secretion of a linked polypeptide is a well-known property of bacterial proregions. Although most bacterial proregions are amino-terminal protein extensions, several carboxyl-terminal peptides have been shown to function in this capacity (*33*). The mechanism by which bacterial proregions serve to enhance secretion appears to be through their capacity to accelerate the folding of the linked polypeptide domain (*33*). Thus, our findings, that elimination of the carboxyl-terminal region of TPO substantially reduces its level of production in mammalian cells, suggest that it serves as a proregion. In many bacterial systems, the presence of the proregion is absolutely essential for production of the corresponding bacterial protease. In contrast to TPO, the carboxyl-terminal "proregion" is a relative enhancer of protein production. Bacterial proregions also act to abolish activity of the linked polypeptide (*34*). It is of interest that mTPO truncation mutants display relative increases in specific activity over those of their full-length homologues. This finding also reinforces the hypothesis that the carboxyl-terminal domain of TPO serves as a proregion.

Proregions have also been described in eukaryotic systems, although their functional role is less well defined. Examples of eukaryotic proregions which enhance or are essential for secretion of their associated protein include the prosequences of prolactin (*35*), transforming growth factor (TGF)-β1 and Activin A (*36*), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF) (*37*), insulin, and von Willebrand Factor (*33*). The mechanism of prosequence activity differs between these proteins, but is similar in some families of proteins. For example, the von Willebrand Factor prosequence directs disulfide bond formation and multimer assembly (*33*). The proregions of AR and HB-EGF prosequences both function as a membrane anchor (*37*). Herein, we have begun to characterize the function of the TPO proregion. While the TPO proregion is not absolutely essential for secretion of the protein, it enhances production substantially.

For our assessment of enhanced secretion, we used two methods: (1) metabolic labeling, immunoprecipitation, and phosphorimaging and (2) ELISA. Each method has potential limitations. Metabolic labeling of TPO is dependent on the number of Met and Cys residues. Thus, the truncated TPO forms are anticipated to incorporate fewer radiolabeled amino acids. All the TPO forms used in these studies have one Met residue and four Cys residues in the receptor binding domain; TPO1−335 has two additional Met residues. The relative increase in the intensity of the TPO1−335 band cannot be accounted for by the additional incorporation of radiolabel, anticipated to be 29%, and this relative intensity was accounted for in our calculations. The second method utilized to detect TPO and truncated TPO isoform production, ELISA, might underestimate the amount of the truncated forms produced if the detecting antibodies are differentially recognized full-length and truncated forms. To account for this potential problem, we used a proteolytically processed form of full-length TPO with an M_r of 18−30 kDa as an ELISA standard, corresponding nicely to the TPO1−152 and TPO1−174 forms we engineered. Thus, we believe both methods accurately evaluate TPO production levels, and the results were highly concordant.

Our experimental conditions may have influenced the outcome of our experiments. Each supernatant was transiently expressed and collected within a 24−72 h period following transfection. To discern if discrepant half-lives of the truncation and native-like muteins could account for the apparent differences in secretion, we measured the half-life of TPO in supernatants from established cell lines. We found no change in activity of either TPO1−335 or TPO1−152, indicating that differences in the survival of the protein in tissue culture do not account for our observation of less efficient production. Additionally, the TPO constructs utilized in our studies have been slightly modified from the native template. All but one of our truncated muteins and none of the glycomuteins contain a pYpH tail, and one of our truncation muteins contains a spacer without TPO residues or glycosylation sites to separate the pYpH region from the receptor binding domain. We have compared the activity and secretion of our TPO1−335 construct with those of the native sequence (lacking a pYpH tail and other modifications), and found no difference in secretion or activity. While it is possible that the spacer region encoded in TPO1−152 could have influenced the stability of the ensuing RNA message, or the secretion or activity of the protein, we believe this is unlikely, given the lack of native residues within this sequence and the relative loss of secretion we observed with progressive truncation of the protein.

We chose to conduct our studies in transformed cell lines that represent normal sites of TPO production. The unique glycan domain appears to function to enhance secretion of the protein in two mammalian fibroblast cell lines and in a hepatoma cell line. The presence of the carboxyl-terminal domain of the protein enhanced TPO secretion in all three cell lines that were tested. The liver is the major site of TPO production in vivo under steady-state conditions (*18*, *19*); this was reinforced by our finding immunoreactive TPO in HepG2 cells prior to their transfection with the TPO expression vectors (see lane 2 in Figure 2B).

Within the hematopoietic growth factor family, glycosylation has been shown to have no effect on secretion of GM-CSF (*38*), and to impact the circulatory half-life (*39*), biosynthesis, secretion, and function of EPO (*40*). While the critical role of glycosylation for the in vivo activity and half-life of EPO is well established (*39*, *41*, *42*), the role of glycosylation of EPO in efficient secretion is more controversial. Wasley and co-workers (*43*) found that metabolic disruption of O-linked and complete N-linked glycosylation had no effect on secretion; however, Delorme and co-workers (*44*) generated glycomuteins which sequentially mutated the three N-linked glycosylation sites as well as the one O-linked glycosylation site and found that while forms with single mutations of N-linked sites and mutation of the O-linked site in combination were as efficiently secreted as native EPO, one double mutation (Q 38,83) and the triply N-linked glycomutein were poorly secreted from COS1 and CHO cells.

We have examined the role of N-linked glycosylation of TPO in the glycan domain, to assess its contribution to secretory efficiency. Elimination of at most four N-linked sites of glycosylation by substitution of N sites with Q did not impact secretion or function. Elimination of all seven potential sites of glycosylation resulted in a modest decrease in the level of secretion (glycomuteins N1−7Q and N1,2,6,-7Q were expressed at just more than half the level of native TPO), indicating that N-linked glycosylation does not account for much of the up to 29-fold loss in the level of secretion between native TPO and truncated TPO (TPO1−152). All but the most substantially mutated glycomutein were active, as were the two truncated constructs lacking all sites for N-linked glycosylation; this finding is consistent with previous observations that the receptor binding domain is necessary and sufficient for TPO activity. It is unlikely that the loss of function of TPO−N1−7Q is due to a direct effect from the mutated residues on receptor binding sites. However, the significant difference in charge caused by mutation of all seven potential glycosylation sites may modify the interaction between the receptor binding and glycan domains and thus indirectly interfere with receptor binding. We used immunologic methods to detect our glycomuteins, as we used for the truncation mutants. The anti-peptide antibodies used for immunoprecipitation of metabolically labeled supernatants are directed against amino-terminal regions of the protein and are hence unlikely to recognize carboxyl-terminal glycomuteins differently from TPO1−335. However, the ELISA uses a polyclonal secondary antibody which may include antibodies to epitopes within the carboxyl-terminal region. Nonetheless, we would anticipate a similar or reduced ability of these antibodies to recognize the glycomuteins rather than *enhanced* ability, and hence, our data may actually underestimate the secretion of the glycomuteins. Therefore, we conclude that N-linked sites of glycosylation have, at most, a modest impact on secretion. The lack of an effect of N-linked glycosylation on secretion, taken together with our findings that progressive truncation results in a progressive loss of secretory efficiency, suggests that the polypeptide sequence, rather than the complex carbohydrate, is essential for this function.

Mutation of the potential N-linked sites of glycosylation in murine TPO resulted in a lower observed molecular mass, confirming that most of these sites are glycosylated in TPO1−335 in our mammalian expression systems. Some of the homologous sites of glycosylation have been experimen-

tally identified in human TPO (*21*); our observations suggest that the murine N-linked glycosylation sites are homologous, and that the seventh potential site of glycosylation in murine TPO (N228), which is not present in the human homologue, is not glycosylated normally.

While we have identified an important function for the unique glycan domain of TPO, the mechanism of its action remains to be elucidated. The carboxyl-terminal region (1) may act as a chaperone to promote structural stability early or late in the process of biosynthesis, assembly, and secretion, (2) may act to protect the protein from degradation, or (3) like bacterial proregions may facilitate the proper folding of the protein. In addition, or alternatively, the carboxyl-terminal region may facilitate transport of the protein through the Golgi apparatus or across the cell membrane, or the glycan domain RNA may function to stabilize the message of the receptor binding domain. We have detected truncated TPO forms and TPO1−335 in lysates of established cell lines, but have been unable to detect any form in lysates of cells transiently expressing TPO or TPO muteins. It is likely that any TPO that fails to traffic normally is rapidly degraded, and that the level of expression is not high enough to be detected with our reagents in transiently transfected cells. While the data we report suggest that the carboxyl-terminal domain of TPO functions as a prosequence, it differs from many prosequences in the following aspects. (1) It is not *absolutely* essential for secretion. (2) There is no convincing evidence that it is cleaved from the receptor binding domain of the hormone in vivo. (3) It does not fully inhibit the function of the receptor binding domain. (4) When TPO1−174 is denatured (in urea), it can slowly refold without the full carboxyl-terminal region (our unpublished observation). In contrast, the prosequences of TGF-β1, activin A, and prolactin (also a member of the hematopoietic cytokine family) are essential for secretion. However, there are other mechanisms, which can be attributed to other prosequences, which may account for the functional activity of the glycan domain of TPO as well. For example, the barnase prosequence appears to provide additional binding sites for molecular chaperones and thus enhances the interaction of the transport machinery of the cell (*33*).

We have identified a new functional role of the carboxyl-terminal domain of TPO and begun to characterize the essential structural elements of this region. This region of murine TPO enhances protein secretion up to 29-fold. The role of O-linked carbohydrate has not been addressed in this paper; however, N-linked glycosylation does not appear to contribute substantially to the function of this region. The mechanism of enhanced secretion and the physiologic significance of this function remain to be elucidated. However, these findings provide another example of a protein dependent on a linked polypeptide region for efficient secretion. Given the few available examples of mammalian proteins containing proregions, it is hoped that a better understanding of the biochemical mechanism by which the carboxyl-terminal domain enhances TPO secretion will provide new insights into this important physiologic process.

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