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Thrombin cleaves recombinant human thrombopoietin: One of the proteolytic events that generates truncated forms of thrombopoietin

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ABSTRACT **A heterogeneity in the molecular weight (M_r) of thrombopoietin (TPO) has been reported. We found several thrombin cleavage sites in human, rat, murine, and canine TPOs, and also found that human TPO undergoes selective proteolysis by thrombin. Recombinant human TPO (rhTPO) was incubated with human platelets in the presence of calcium ions to allow the generation of thrombin, and was cleaved into low M_r peptide fragments. The cleavage was completely inhibited by hirudin, indicating that the proteolysis was mediated by thrombin. In a platelet-free system, analyses of thrombin cleavage by immunoblotting using anti-human TPO peptide antibodies revealed that the four major thrombin-cleaved peptide fragments were selectively generated depending on the digestion time. The amino acid sequences of the thrombin-polypeptides were further analyzed, and two major thrombin cleavage sites were determined. One of them was at AR^{191}-T^{192} in the C-terminal domain of TPO, and thrombin cleaved first at this site. The other site at GR^{117}-T^{118} in the N-terminal domain was subsequently cleaved by prolonged thrombin digestion. As a result, the biological activity of TPO was modulated. The generation of truncated forms of TPO by thrombin may be a notable event in view of the platelet-related metabolism of TPO.**

Endogenous thrombopoietin (TPO), c-Mpl ligand has an essential role in megakaryopoiesis and platelet production (1). The presence of truncated forms of TPO in thrombocytopenic plasma from various species has been noted by several groups in their studies on the purification of TPO (2–5), and the electrophoretically estimated molecular weight (M_r) of these forms ranged from 18,000 to 36,700. In addition, the heterogeneity in M_r, ranging from 17,000 to 43,000, was also found among native TPOs derived from cell culture supernatants of rat hepatoma cells (6). Although alternative splicing of TPO mRNA may contribute to the heterogeneity of TPO, a multiplicity of biologically active TPOs suggests that the various forms may largely result from proteolysis. The proteolytic activity could be present in the plasma; however, the generation of such various forms of TPO could not arise merely during several stages of purification (7).

Recent findings indicate that circulating platelets may regulate the plasma concentration of TPO (3). We and others have shown that platelets have functional receptors (c-Mpl) on their surface. Thus, TPO enhances the degree of activation of platelets by various agonists or by shear stress, and induces protein tyrosine phosphorylation of numerous proteins in platelets such as Jak2, Tyk2, Shc, c-Cbl, Stat3, Stat5, and Crkl (8–11). Kuter *et al.* (3, 12) have shown that plasma TPO levels increased in Busulfan-treated thrombocytopenic rabbits and that infusion of platelets into the rabbits lowered the plasma levels of TPO. Fielder *et al.* (13) have shown that normal mouse platelets adsorb TPO, whereas platelets from c-mpl −/− mice did not, indicating that the adsorption of TPO by platelets is mediated through the specific TPO receptor (c-Mpl). They also demonstrated that platelet-rich plasma (PRP) but not platelet-poor plasma (PPP) degraded radiolabeled TPO, suggesting that the cleavage is platelet-dependent. To date, no natural protease has been identified to cleave TPO specifically.

A number of potential proteolytic cleavage sites exist in the human TPO. Among them, we found several thrombin cleavage sites (14) in human (2, 15, 16), rat (4, 17), murine (16, 18), and canine (16) TPOs. Thrombin is a multifunctional serine protease acting in the regulation of the coagulation cascade as a key factor, and it also activates various cells such as platelets, smooth muscle cells, and endothelial cells (19). Importantly, guinea-pig megakaryocytes were shown to generate thrombin from prothrombin, and proplatelet formation was affected by thrombin (20). Moreover, thrombin plays functional roles in the regulation of megakaryopoiesis (21). Because thrombin can be formed on the surface of platelets or microparticles derived from platelets, we postulated that thrombin may cleave TPO. In this study, we demonstrate that recombinant human TPO (rhTPO) undergoes selective proteolytic cleavage in the presence of platelets plus calcium ions to allow the generation of thrombin, and that purified thrombin also cleaves TPO. Further, the major cleavage sites in TPO have been determined. These findings may be of significant importance to better understand the metabolism of TPO and the regulation of its plasma level by platelets.

MATERIALS AND METHODS

Proteins. rhTPO was expressed in Chinese hamster ovary cells, and purified to homogeneity by the Production Technology Group of Kirin Brewery Co., Ltd. Purified human plasma thrombin was generously provided by Green Cross (Osaka). Recombinant hirudin was kindly provided by Japan Energy (Tokyo).

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Abbreviations: DPBS, Dulbecco's phosphate buffered saline; ECL, enhanced chemiluminescence; MAP, multiple antigenic peptide; PPP, platelet-poor plasma; PRP, platelet-rich plasma; PVDF, polyvinylidene difluoride; TPO, thrombopoietin; rhTPO, recombinant human TPO.

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Platelet Preparation and Digestion of rhTPO with Platelet Suspension. Human blood from healthy volunteers was drawn by venipuncture into 1/10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. PRP was prepared by centrifuging the whole blood at 200 × *g* for 20 min and aspirating PRP. Prostaglandin E1 (1 μM; Sigma) was added from a stock solution in absolute ethanol (1 mM). The PRP was centrifuged at 800 × *g* to form a soft platelet pellet. The pellet was resuspended in a modified Hepes·Tyrode buffer (129 mM NaCl/8.9 mM $NaHCO_3$/0.8 mM KH_2PO_4/0.8 mM $MgCl_2$/5.6 mM dextrose/10 mM Hepes, pH 7.4) at a concentration of 3×10^8 platelets per ml. Then, digestion of rhTPO by the platelet suspension was performed as described in detail in the figure legends. Briefly, rhTPO was added to a platelet suspension (3×10^8 platelets per ml in a nominally calcium-free modified Hepes·Tyrode buffer) and incubated for various times at 37°C in the presence or absence of 1 mM calcium ions. EGTA (1 mM) was suitably added to the incubation mixture to block the generation of thrombin by calcium ions. To confirm that the generation of truncated rhTPO was mediated by thrombin, we incubated rhTPO with the platelet suspension in the presence of recombinant hirudin (10 units per ml) plus 1 mM calcium ions. After the incubation, the resultant suspensions were lysed by adding 2× concentrated SDS/PAGE buffer (final concentrations of 10% glycerol/1% SDS/1 mM DTT/50 mM Tris·HCl, pH 6.8/1 mM EDTA/0.002% bromophenol blue), heated for 3 min at 95°C, and then subjected to 7.5–15% gradient SDS/PAGE under reducing conditions. Proteins were visualized by immunoblotting, as described below.

Digestion of rhTPO with Thrombin in Platelet-Free Incubation. rhTPO (50 μg/ml) was incubated in the presence or absence of human thrombin (5 units per ml) in Dulbecco's phosphate buffered saline (DPBS; Nissui Pharmaceuticals, Tokyo) at 37°C for various periods as indicated in the figure legends. The proteolytic reaction was terminated by the addition of one-fourth volume of 5× concentrated SDS/PAGE buffer followed by heat treatment for 3 min at 95°C. The aliquot was subjected to SDS/PAGE for silver staining or Western blotting.

Antibodies to TPO and Synthesized TPO Peptides. Anti-rhTPO rabbit IgG fraction (anti-rhTPO Ab) was obtained from antiserum (22) by use of a Protein A Hyper D column (BioSepra, Marlborough, MA). To prepare anti-TPO peptide rabbit antibodies (anti-TPO peptide Abs), we selected six peptide regions, D^{8}LRVLSKLLRDSHVLHSRLSQ^{28} (HT1), S^{47}LGEWKTQMEETKAQD^{62} (HT2), L^{108}GTQLPPQGRTTAHKDPNA^{126} (HT3), N^{172}ELPNRTSGLLETNFTASA^{190} (HT4), S^{262}LPPNLQPGYSPSPTHPPTGQYT^{284} (HT5), and P^{306}SAPTPTPTSPLLNTSYTHSQNLSQEG^{332} (HT6), as suitable peptide antigens from the amino acid sequence of TPO. Then, each quadruple-stranded multiple antigenic peptide (MAP) was synthesized by a model 431A peptide synthesizer (Perkin–Elmer) by the procedure of Tam (23). Rabbits were then immunized eight times with 100 μg of a given MAP, and antisera were collected. Each anti-TPO peptide Ab was purified by use of a Sulfo-Link affinity column (Pierce), to which a Cys residue at the C terminus of the monomeric peptide was coupled. In brief, a solution of each monomeric peptide was bonded via a Cys residue during a 15-min incubation to Sulfo-Link coupling gel previously equilibrated with the coupling buffer (5 mM EDTA/50 mM Tris·HCl, pH 8.5). Then, the gel was washed with the same buffer, and 0.05 M L-Cys-HCl was added for blocking over a 15-min period. The antiserum was applied to the peptide antigen column pre-equilibrated with 50 mM phosphate buffer (pH 8.0) containing 150 mM NaCl and 0.05% sodium azide, and washed with the same buffer. The adsorbed monomeric antigen peptide-specific anti-TPO peptide Ab was then eluted with 0.1 mM citrate buffer (pH 3.0) and neutralized with a 0.1 M carbonate buffer (pH 9.9). The coupling reactions and purification were carried out at 25°C.

Gel Electrophoreses and Western Blot Analyses. The reduced samples prepared in a SDS/PAGE buffer were subjected to SDS/PAGE using a 10–20% precast gradient gel (Daiichi Pure Chemicals, Tokyo). Prestained protein markers (New England BioLabs) were used for electrophoretic estimation of M_r. After electrophoresis, the proteins were visualized by silver staining (2D-Silver Stain II, Daiichi Pure Chemicals), or transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). Protein transfer was carried out for 2 hr at a constant current of 200 mA with a semi-dry electroblotter (model HEP-1, Owl Scientific, Woburn, MA) by using solutions of the anolyte (0.3 M Tris/20% methanol, pH 10.4), the transfer membrane solution (25 mM Tris/20% methanol, pH 10.4), and the catholyte (25 mM Tris/40 mM aminocaproic acid/20% methanol, pH 10.4). The blots were washed with 20 mM Tris·HCl/0.5 M NaCl, pH 7.5 (TBS) containing 0.1% Tween 20 (TTBS) for 10 min, and washed with TBS for 10 min. The blots were then treated with 0.1% $NaIO_4$ for 30 min for removal of sugar chains to obtain maximal detection with each anti-TPO peptide Ab. After blocking with a gelatin hydrolysate (Boehringer Mannheim) in TTBS (GTTBS) for 60 min followed by subsequent blocking with Block Ace (Dainippon Pharmaceutical, Osaka) for 60 min, each blot was incubated for 15 hr with 1 μg/ml of anti-rhTPO or anti-TPO peptide Ab, and then washed twice with TTBS. After that, the blots were incubated for 1 hr with goat anti-rabbit biotinylated IgG (Dako), washed with TTBS twice for 10 min each time, and treated with peroxidase-conjugated streptavidin (Dako) for 1 hr. After five washes with TTBS, the proteins were detected by an enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham). The Western blot analysis described here was carried out at 25°C except for the first antibody reaction, which was performed at 4°C. All antibodies and peroxidase-conjugated streptavidin were prepared in a mixture solution of Block Ace and GTTBS (1:1).

Cell Proliferation Assay for Measurement of the TPO Activity. To measure *in vitro* TPO activity of thrombin-cleaved rhTPO, we conducted a cell proliferation assay using FDCP-hMpl5 cells (11), which were genetically engineered to constitutively express human c-Mpl, as previously reported (24). Briefly, the thrombin digestion was terminated by the addition of hirudin without a reducing reagent. Each diluted aliquot (0.1 ml) was added to a suspension (0.1 ml) of the exponentially growing FDCP-hMpl5 cells (2.5×10^4 cells per ml), and incubated on a 96-well microplate in Iscove's modified Dulbecco's medium with 10% FCS in 5% CO_2 for 72 hr at 37°C. Thereafter, a 20-μl mixture of a tetrazolium compound [3-(4,5-dimethylthiozol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and phenazine methosulfate as a coupling reagent (CellTiter 96 AQ Assay, Promega) was added into each well and the cells were further incubated for 4 hr at 37°C. The cell growth was determined by measuring the absorbance at 492 nm.

Identification of the Thrombin-Cleaved Peptide Fragments. For preparation of a sufficient amount of thrombin-digested peptide fragments for the amino acid sequence analysis, a higher concentration of rhTPO (900 μg/ml), in comparison with that used in the time course experiments described above, was incubated at 37°C for 16 hr in DPBS in the presence of a concentration of thrombin (9 units per ml). The resultant aliquot was analyzed on SDS/PAGE, and showed the identical cleavage profile to that obtained by incubation of rhTPO (50 μg/ml) with thrombin (5 units per ml) for 16 hr at 37°C as shown in Figs. 2 and 3. The major thrombin-cleaved peptide fragments were transferred onto a PVDF membrane after SDS/PAGE was stained with Coomassie brilliant blue R-250, and subsequently destained with methanol before the analysis. Thereafter, each thrombin-cleaved peptide fragment was sub-

jected to Edman degradation followed by amino acid sequencing with a model 492 gas-phase protein sequencer (Perkin–Elmer) equipped with an on-line phenylthiohydantoin amino acid analyzer. The amino acid sequence consisting of more than 20 amino acid residues from the N-terminal end of each fragment was determined. Peptide fragments were also subjected to an amino acid composition analysis to determine the C-terminal end by the AccQ Tag amino acid analysis system using a fluorescent derivatizing reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, Millipore, Waters; ref. 25).

RESULTS

Human Platelet Suspensions Cleave rhTPO. In view of the recent report that murine TPO is cleaved by PRP but not by PPP (13), we first examined whether human platelets also cleave rhTPO. PRP was centrifuged once to remove a portion of plasma, and the platelets were resuspended in a buffer containing 1 mM $CaC1_2$ and exogenous rhTPO. There was a time-dependent accumulation of a 34-kDa protein and another weak diffuse band (46–69 kDa) recognized by the anti-rhTPO Ab (Fig. 1*A*). Under the conditions, there was a significant contamination by plasma proteins, including prothrombin, in the platelet suspensions. Accordingly, we suspected that rhTPO is cleaved by thrombin generated on the surface of platelets in the presence of calcium ions and that the 34-kDa protein represents the cleaved peptide fragment of rhTPO. To directly test this possibility, rhTPO was incubated with platelet suspensions in the presence or absence of calcium ions, and we found that the 34-kDa protein appeared in the presence of 1 mM Ca^{2+} (Fig. 1*B*). Even in the presence of 1 mM Ca^{2+}, the appearance of the 34-kDa and other diffuse TPO-associated proteins was inhibited by the addition of hirudin, a specific inhibitor of thrombin (26–28). These proteins corresponded to peptide fragments generated by the incubation of rhTPO with purified thrombin (Fig. 1*B*). Thus, it is likely that platelet suspensions cleave rhTPO and that the cleavage is mediated by thrombin.

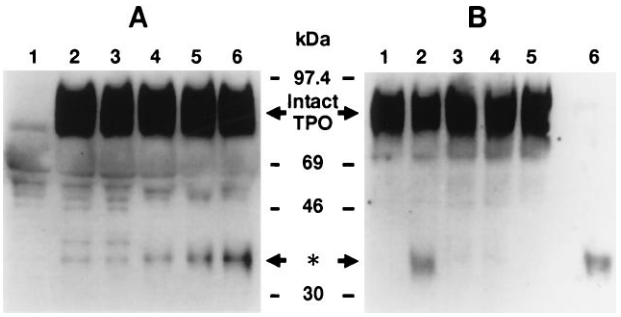

FIG. 1. Cleavage of rhTPO by platelet suspensions. (*A*) Time course. Platelet pellets were resuspended in a nominally calcium-free modified Hepes·Tyrode buffer at a concentration of 3×10^8 platelets per ml. RhTPO (1 μg/ml) and 1 mM calcium ions were or were not added to the platelet suspension, and incubation was conducted for various times at 37°C. The platelets were then lysed by the addition of 2× concentrated SDS/PAGE buffer, and subjected to 7.5–15% gradient SDS/PAGE. Proteins were then electroblotted onto a PVDF membrane, stained by anti-rhTPO Ab, and detected by the ECL method, as described. Prestained protein markers were obtained from Amersham. Lanes: 1, platelet lysate only without exogenous TPO; 2, sample immediately lysed after the addition of rhTPO (1 μg/ml) and 1 mM calcium ions; 3–6, samples lysed 1, 10, 30, and 60 min, respectively, after those additions. The position of the intact rhTPO and the 34-kDa protein band of interest (*) are indicated by the arrows. (*B*) The cleavage of rhTPO is inhibited by hirudin and requires calcium ions. Platelets were lysed by the addition of 2× concentrated SDS/PAGE buffer immediately or after incubation for 1 hr at 37°C with rhTPO (1 μg/ml) in the presence or absence of 1 mM calcium ions. Lysed samples were subjected to 7.5–15% gradient SDS/PAGE. RhTPO was detected as described in *A*. Lanes: 1, immediately lysed sample; 2, 1-hr incubation in the presence of calcium ion (1 mM); 3, 1-hr incubation without the addition of calcium ions; 4, 1-hr incubation with EGTA (1 mM); 5, 1-hr incubation in the presence of calcium ion plus hirudin (10 units per ml); 6, free rhTPO incubated with thrombin (10 units per ml).

Transition of Thrombin-Generated Polypeptides Derived from rhTPO and *in Vitro* TPO Activity. To further characterize the cleavage of rhTPO by thrombin, we examined the fragmentation of rhTPO by exogenous human thrombin in platelet-free systems. In preliminary experiments, we observed by SDS/PAGE that thrombin digestion generated various peptide fragments. Therefore, the first experiment was performed to determine whether such generation of multiple peptide fragments was dependent on the digestion time. The digestion was performed for periods of from 0 to 64 hr. To compare relative contents of peptide fragments on SDS/PAGE, we conducted silver staining for nonspecific protein detection separately from immunochemical staining (Fig. 2*A*). The incubation of full-length rhTPO (intact rhTPO) in the presence of thrombin resulted in the generation of multiple peptide fragments, and the content of each of these polypeptides changed depending on the incubation period. A 34-kDa protein appeared within 0.5 hr, as was found by the incubation of rhTPO with platelets. Then, the maximal detection was obtained after around 4 hr of incubation, and gradually decreased in a time-dependent manner. At 64 hr of incubation, silver-detectable intact rhTPO was almost absent. The thrombin-cleaved peptide fragments were more clearly visualized on immunoblots probed with anti rhTPO Ab, as shown in Fig. 2*B*. The major thrombin-cleaved peptide fragments were designated as FA (around 48 kDa), FB (around 34 kDa), FC (around 25 kDa), and FD (below 10 kDa). Both of the FA and FB appeared within 0.5 hr; the FA gradually increased, whereas the FB decreased. In addition, the much lower M_r peptide fragments (FC and FD) became detectable after 4 or more hr of incubation. The appearance of FC and FD corresponded to the reduction in the amount of FB. The generation of such peptide fragments was completely inhibited by the

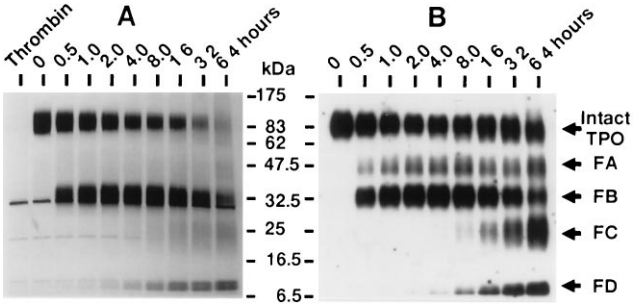

FIG. 2. Time course analysis of thrombin cleavage of rhTPO in a platelet-free incubation. Thrombin cleavage of rhTPO was analyzed by SDS/PAGE under reducing conditions. Proteins were visualized by silver staining for nonspecific protein detection (*A*), and also by immunoblotting using anti-rhTPO Ab (*B*). To examine the time-dependent transition of the formation of thrombin-digested components, rhTPO (50 μg/ml) was incubated at 37°C in the presence of thrombin (5 units per ml). The reaction was terminated at 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32, or 64 hr by the addition of SDS/PAGE sample buffer and heat treatment at 95°C for 3 min. (*A*) A 10-μl aliquot after the digestion containing rhTPO (400 ng) and thrombin (0.04 units) was applied to each lane for silver staining. As a control, thrombin (0.04 units) without rhTPO was charged onto the left lane. The major band (above 30 kDa) in the thrombin preparation was α thrombin. (*B*) A 0.025-μl aliquot containing rhTPO (1 ng) and thrombin (100-μ units) was applied to each lane and detected by the ECL method. The cleavage products, having a M_r lower than that of the intact TPO were classified into four major ones (FA, FB, FC, and FD). These peptide fragments were transferred onto a PVDF membrane, and their N-terminal amino acid sequence and amino acid composition were analyzed (Fig. 5).

addition of hirudin (1 unit per ml) to the incubation mixture of rhTPO (50 μg/ml) and thrombin (5 units per ml) at 37°C (data not shown).

To further classify the domains of thrombin-cleaved peptide fragments, the thrombin-digested samples were probed by six different anti-TPO peptide Abs against HT1, HT2, HT3, HT4, HT5, and HT6 peptide regions (Fig. 3). The results indicated that peptide fragment FA was stained with anti-HT5 and anti-HT6 Abs in samples taken after ≈0.5–64 hr of incubation, and was also reactive with anti-HT4 Ab after 64 hr. This suggests that the FA consisted mainly of amino acid residues located in the C-terminal domain including HT5 and HT6 regions. On the other hand, FB fragment was positively stained with anti-HT1, anti HT2, anti-HT3, and anti-HT4 Abs in samples taken from 0.5 to 64 hr of incubation, suggesting that FB consisted of amino acid residues from around the N-terminal end to A^{190}. Therefore, thrombin cleaved rhTPO into two fragment species of FA and FB, indicating that a thrombin cleavage site was in a peptide region between HT4 and HT5. FC was positively stained with anti-HT4 Ab, but was unreactive with the others. Accordingly, the FC might contain a peptide region between HT3 and HT4. The FD was positive to both anti-HT1 and anti-HT2 Abs at the incubation periods of 4–64 hr, and was also stained strongly by anti-HT3 Ab over all incubation periods. These results mean that the low M_r peptide fragments were derived mainly from the N-terminal region of rhTPO. The appearance of FD corresponded to the reduction in the amount of FA and FB. Additionally, a peptide fragment consisting of FA and FC that was reactive with anti-HT4 was detected only after 64 hr of incubation, suggesting that FD was generated mainly from FB. Although there were several minor cleavage components [e.g., anti-HT6 positive FD after 8.0 hr of incubation (Fig. 3*F*) and anti-HT4 positive protein between intact rhTPO and FA at 64 hr of incubation (Fig. 3*D*)], the transition of various species of peptide fragments generated by thrombin indicated that the thrombin cleavage first occurred selectively at around A^{190} in the C-terminal region of intact rhTPO generating FA and FB, and the latter was then cleaved to form FC and FD.

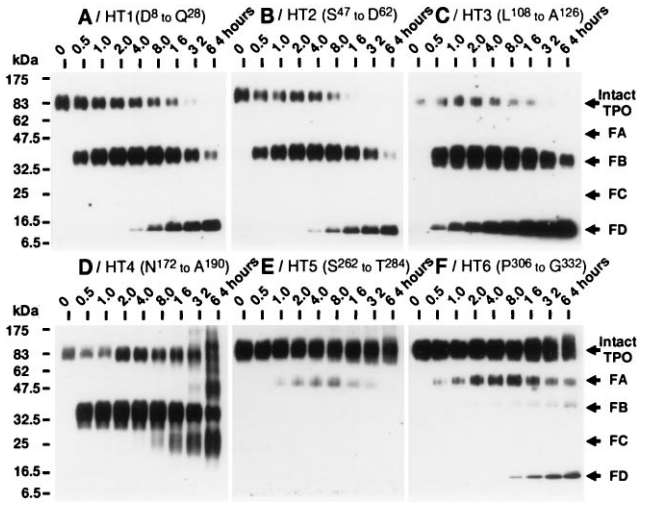

FIG. 3. The transition of domains in thrombin-cleaved components derived from intact rhTPO. Aliquots of thrombin-digested rhTPO, obtained at the same time intervals as described in the legend to Fig. 2, were further analyzed on immunoblots probed with various anti-TPO peptide Abs. Since the sensitivity to the antigen varied among the anti-TPO peptide Abs, appropriate amounts of the aliquots were applied onto SDS/PAGE gels to achieve maximal detection of each antigen. After incubation at 37°C for the times indicated, TPO peptide fragments on each immunoblot were visualized by the ECL method. These Western blots were proved by anti-HT1 Ab, 1 ng of rhTPO per lane (*A*); anti-HT2 Ab, 5 ng of rhTPO per lane (*B*); anti HT3 Ab, 100 ng of rhTPO per lane (*C*); anti-HT4 Ab, 50 ng of rhTPO per lane (*D*); anti-HT5 Ab, 500 ng of rhTPO per lane (*E*); anti-HT6 Ab, 500 ng of rhTPO per lane (*F*). The peptide domains recognized by the anti-TPO peptide Abs here are shown in Fig. 5.

In addition to the analysis of peptide domains, the FDCP-hMpl5 cell proliferation assay revealed that the *in vitro* TPO activity was changed by thrombin digestion as shown in Fig. 4. The total activity of each batch obtained from the time course experiments, a mixture of thrombin-cleaved TPO-peptides, was gradually increased as FB was generated, and reached the maximal activity after 1–4 hr of incubation. The activity then decreased to the basal level in a time-dependent manner, as low M_r products (FC and FD) appeared.

Identification of Thrombin Cleavage Sites. The thrombin sites in proteins were reported by Chang (14). As shown in Fig. 5, we predicted six thrombin sites in human TPO, and they were designated as "Tbn" sites. To identify the actual thrombin cleavage sites, we subjected each major thrombin-cleaved peptide fragment to N-terminal amino acid sequencing together with amino acid composition analysis for the determination of the C-terminal end. As shown in Fig. 5, the FA, FB, FC, and FD fragments were revealed to be peptides consisting of amino acid residues from T^{192} to G^{332}, S^1 to R^{191}, T^{118} to R^{191}, and S^1 to R^{117}, respectively; whereas intact rhTPO was confirmed to be S^1 to G^{332}. In other words, the actual major thrombin cleavage sites in rhTPO were identified as GR^{117}–T^{118} and AR^{191}–T^{192}. Therefore, all predicted thrombin sites were not cleaved under our experimental conditions, though there may be other minor thrombin cleavage sites under different conditions (e.g., denaturing conditions or not, digestion time, pH, concentrations of thrombin and rhTPO, the degree of autolysis of thrombin during digestion, etc.).

DISCUSSION

We found that rhTPO is cleaved in human platelet suspensions containing 1 mM Ca^{2+} (Fig. 1*A*). Because the cleavage of rhTPO in the platelet suspensions was inhibited by hirudin, a thrombin-specific inhibitor, thrombin generated *in situ* seems to be responsible for the cleavage of rhTPO (Fig. 1*B*).

However, thrombin is known to activate other proteases (e.g., protein C). Therefore, naturally we turned to a platelet- and plasma-free system to further investigate the thrombin-induced cleavage of rhTPO. In this study, the major selective thrombin cleavage sites were determined as AR^{191}–T^{192} in the C-terminal domain and GR^{117}–T^{118} in the N-terminal domain of TPO. The sequencing analysis revealed that FC fragment contained the C-terminal region of HT3, whereas FC was not

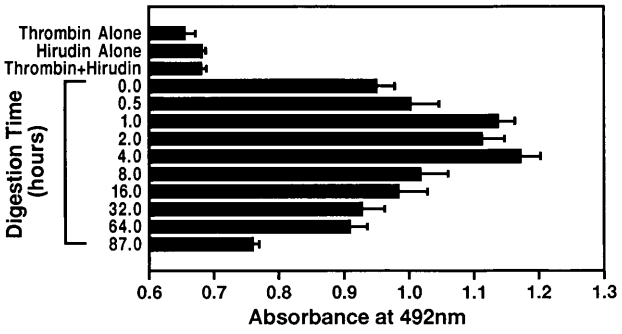

FIG. 4. *In vitro* TPO activity after thrombin digestion. RhTPO (50 μg/ml) was incubated at 37°C in the presence of thrombin (5 units per ml). The reaction was terminated at 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32, 64, or 87 hr by the addition of hirudin (1 unit per ml). Without the addition of rhTPO, the results at 0-hr incubation of thrombin alone, hirudin alone, and thrombin plus hirudin were indicated as negative control. The samples were subjected to the FDCP-hMpl5 cell proliferation assay as described. The data (absorbance at 492 nm) represent the mean ± SD from triplicate assays.

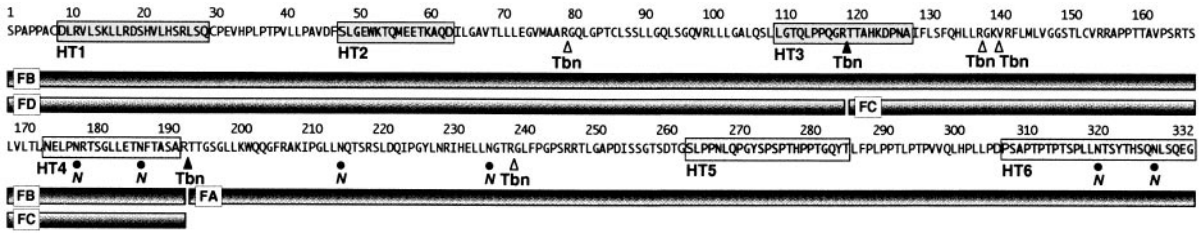

FIG. 5. Antigenic peptide domains recognized by anti-TPO peptide Abs and thrombin cleavage sites of rhTPO. The selected antigenic peptide domains of HT1 (D^{8} to Q^{28}), HT2 (S^{47} to D^{62}), HT3 (L^{108} to A^{126}), HT4 (N^{172} to A^{190}), HT5 (S^{262} to T^{284}) and HT6 (P^{306} to G^{332}) are denoted by shaded boxes. Each amino acid residue in the sequence is shown in the one-letter amino acid code. *N*-glycosylation sites are indicated by a closed circle with an *N*. Thrombin cleavage sites (14) are indicated along a TPO amino acid sequence. In human TPO, there is no site of [P4]-[P3]-[Pro]-[Arg or Lys]-[P1′]-[P2′], where P3 and P4 are hydrophobic amino acids and P1′ and P2′ are non-acidic amino acids. On the other hand, the predicted thrombin sites, which are [P2]-[Arg or Lys]-[P1′], where P2 or P1′ is Gly and the [Arg or Lys]-[P1′] bond is cleaved, were found in human TPO at AR^{78}–G^{79}, LR^{136}–G^{137}, GK^{138}–V^{139}, and TR^{237}–G^{238}. In addition, the AR^{191}–T^{192} is one of the other predicted thrombin cleavage sites containing [Ala]-[Arg]-[P5], where the [Arg]-[P5] bond is cleaved, as reported to occur in fibrinogen, chymotrypsinogen A, and antibody λ chain (14). These predicted sites were designated as "Tbn" sites, indicated by triangles. Among them, N-terminal amino acid sequence analyses revealed actual thrombin cleavage sites to be at GR^{117}–T^{118} and AR^{191}–T^{192}, both indicated by closed triangles. The N-terminal amino acid sequences obtained from the major thrombin-cleaved peptide fragments (FA, FB, FC, and FD) and intact rhTPO were as follows: T^{192}TGSGLLKWQQGFRAKIPGL, X(or S^{1}) PAPPAX(or C)DLRVLSKLLRDSH, T^{118}TAHKDPNAIFLSFQHLLRGK, X(or S^{1}) PAPPAX(or C)DLRVLSKLLRDSH, and X(or S^{1}) PAPPAX(or C)DLRVLSKLLRDSH, respectively. Additionally, amino acid composition analysis was conducted to determine each C-terminal end. All results indicated that the major peptide fragments (FA, FB, FC, and FD) consisted of T^{192} to G^{332}, S^{1} to R^{191}, T^{118} to R^{191}, and S^{1} to R^{117}, respectively, as indicated by the open boxes. Both thrombin sites found in human TPO (2, 15, 16) (GR^{117}–T^{118} and AR^{191}–T^{192}) are conserved among TPOs, including mice (16, 18) (GR^{117}–T^{118} and AR^{191}–T^{192}), rats (17) (GR^{117}–T^{118} and AR^{191}–T^{192}), and dogs (16) (GR^{115}–T^{116} and AR^{189}–T^{190}).

strongly recognized by anti-HT3 Ab. It is possible that affinity-purified anti-HT3 Ab may be more reactive against the N-terminal region of the antigen, since it is structurally freer than the C-terminal end of the quadruple-stranded MAP linked together by Lys residues. The analysis of thrombin cleavage analyses by use of anti-TPO peptide Abs revealed that thrombin cleaved rhTPO at selective sites in a discriminated order. Interestingly, thrombin selectively cleaved at AR^{191}–T^{192} of rhTPO first, but did not cleave simultaneously at another site, GR^{117}–T^{118}. We cannot conclude at present whether the removal of the C-terminal polypeptide by cleaving at AR^{191}–T^{192} is structurally required prior to the cleavage at GR^{117}–T^{118}. The result of a test using a truncated TPO rather than a full-length TPO as the initial substrate of thrombin indicated that AR^{191}–T^{192} was hard to be cleaved under nondenaturing conditions (data not shown). It has been predicted that the N-terminal half domain of TPO has a bundle structure consisting of four α-helices with a hydrophobic core based on the analogy with the structure of erythropoietin (29). This may be one of the reasons that the GR^{117}–T^{118} thrombin site in the N-terminal domain of TPO was more resistant against proteolytic cleavage than the AR^{191}–T^{192} site. Since platelets have receptors for thrombin as well as for TPO, it is possible that the association with platelets may affect the structure of rhTPO, and such conformational change of the protein may lead the GR^{117}–T^{118} site to be cleaved easier by thrombin *in vivo*. Taken together, as summarized in Fig. 6, a TPO[1–191] (consisting of amino acid residues from S^{1} at the N-terminal end to R^{191}) was formed first through the cleavage at AR^{191}–T^{192}, and thrombin subsequently cleaved it at the GR^{117}–T^{118}. Moreover, the biological activity of rhTPO was modulated by thrombin cleavage (Fig. 4), though the activity of each thrombin-generated peptide was not individually examined in this study. The *in vitro* TPO activity was increased as FB was generated, indicating that the generation of TPO[1–191] (30) raised the *in vitro* activity. On the contrary, the further thrombin cleavage at GR^{117}–T^{118} reduced or destroyed it. Two disulfide bonds of C^{7}–C^{151} and C^{29}–C^{85} have been identified in rhTPO, and both disulfide loops are essential for exhibiting the TPO activity (30). It should be noted that the peptide fragments of FD (S^{1} to R^{117}) and FC (T^{118} to R^{191}) were still connected to each other via disulfide bonds in the biological assay, whereas they migrated separately on SDS/PAGE gels under reducing conditions. Therefore, further analysis should be conducted to comprehend better how thrombin cleavage affects the biological activity of TPO.

To date, the biochemical information of the native plasma TPO is not sufficient to allow complete understanding about the proteolytic processing that generates truncated TPOs. The molecular masses estimated by SDS/PAGE of porcine (2), ovine (3), rat (4), and canine (5) plasma TPOs was reported to be 28–32 kDa and 18–20 kDa, 31.2 kDa and 36.7 kDa, 19 kDa, and 25 kDa and 31 kDa, respectively. Thrombin cleavage sites exist in rat, murine, and canine TPOs as well as in human TPO. However, the proteolysis by thrombin cannot account for the generation of all the reported purified TPO species in the light of their M_r. Some mechanisms other than the thrombin cleavage may be required for the generation of truncated species around 20 kDa. This supports the notion that other specific proteolytic enzymes or mechanisms may exist and process TPO protein (1, 4, 7).

The most vital point in this study is that thrombin derived from a washed platelet preparation is capable of cleaving TPO. The finding in this study must be taken into account in future studies to examine the "metabolism" of TPO by platelets. Recently, we have reported that the circulating TPO in human

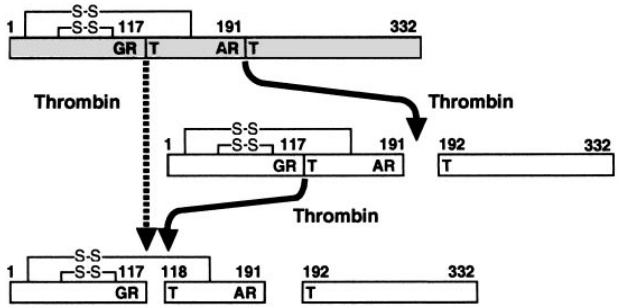

FIG. 6. Schematic thrombin cleavage of rhTPO. From the results of the time course analysis of the major thrombin–cleavage peptide fragments, rhTPO was cleaved at AR^{191}–T^{192} first, and TPO[1–191] was generated. Subsequently, the generated TPO[1–191] was also cleaved by prolonged thrombin digestion at GR^{117}–T^{118} in the N-terminal domain. The biological activity of the resultant proteins are consequently modulated by the thrombin cleavage. Thrombin may directly cleave rhTPO at GR^{117}–T^{118} without the cleavage at AR^{191}–T^{192}, as indicated by the dotted arrow; however, structurally this site is more resistant against thrombin cleavage than the AR^{191}–T^{192} site. Disulfide bonds (C^{7}–C^{151} and C^{29}–C^{85}) are indicated by S–S loops.

plasma, which was not purified, was mainly 80 kDa (31). This finding suggests that TPO undergoes proteolysis under some specific conditions. As previously reported (20, 21), it is possible that thrombin locally modulates the activity of TPO and thus plays a role in megakaryopoiesis.

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