Thrombopoietin-induced Expression of the Glycoprotein IIb Gene Involves the Transcription Factor PU.1/Spi-1 in UT7-Mpl Cells

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Thrombopoietin (TPO) is the major regulator of proliferation and differentiation of megakaryocytes and their progenitors. These actions can be reproduced in the human megakaryoblastic cell line UT7 into which the murine TPO receptor, c-Mpl, was introduced. In these cells, TPO enhanced the expression of the specific megakaryocytic marker integrin glycoprotein (GP) IIb-IIIa while decreasing the expression of erythroid genes (Porteu, F., Rouyet, M.-C., Coccult, L., Benit, L., Charon, M., Picard, F., Gisselbrecht, S., Souyri, M., and Dusanter-Fourt, I. (1996) Mol. Cell. Biol. 16, 2473–2482). We have now analyzed the effect of TPO on the transcriptional activity of the GPIIb promoter in these cells. Using transient transfection assays of a series of human GPIIb promoter fragments, we delineated a TPO-responsive element within the previously reported enhancer region of the promoter. Although this enhancer included cis-acting elements necessary for lineage-restricted expression of GPIIb in vitro and in vivo. Sequences crucial for tissue-specific expression of the GPIIb gene were identified using serial deletions of promoter constructs introduced into megakaryocytic cells. Studies showed that the two associated GATA/Ets-binding sites present in the human or rat GPIIb promoter are necessary for full promoter activity, with a dominant participation of the distal GATA/Ets motif (4, 8, 9). This distal GATA/Ets motif is located in an enhancer region that is active in both erythroid and megakaryocytic cell lines (11). A short enhancer-less GPIIb promoter fragment was also shown to be active in megakaryocytic cells in vitro only. This short 100-bp-long promoter was transactivated in non-megakaryoblastic HeLa cells when co-transfected with the GATA-1, Ets-1, or Fli-1 expression vector, suggesting the positive role of these factors in regulating the expression of the GPIIb gene in megakaryocytes (8, 13). However, although a number of Ets factors including Ets-1, Ets-2, Fli-1, and PU.1 were reported to be present in megakaryocytes, it is still unclear which Ets factors bind to the various GPIIb Ets-binding sites (EBs) and which of these factors are essential for GPIIb promoter activation in vivo. Tissue-specific expression of the GPIIb gene was also shown to depend on unidentified factors that bind to a repressor domain in the proximal part of the promoter. These factors specifically suppress the expression of the GPIIb gene in non-megakaryocytic cells (4, 14).

While GPIIb gene expression is restricted to megakaryocytic cells, its expression is also regulated along with megakaryocytic differentiation. Only a limited number of studies analyzed the promoter elements that participate in this differentiation-dependent expression of the GPIIb gene. These studies always

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1 The abbreviations used are: GP, glycoprotein; hGPIIb, human glycoprotein IIb; bp, base pair(s); EBS, Ets-binding site; TPO, thrombopoietin; GM-CSF, granulocyte-monocyte colony-stimulating factor; EPO, erythropoietin; EMSAs, electrophoretic mobility shift assays.
used erythromegakaryocytic cell lines induced to differentiate in the presence of phorbol esters. Interestingly, in these chemically induced differentiation models, regulatory elements that participated in the temporal expression of GPIIb were identified and located at the same positions as the tissue-specific positive and negative sequences (15). However, the identity of the factors physiologically important in the regulated expression of GPIIb in vivo awaits further investigation.

The cloning of thrombopoietin (TPO) and its receptor, c-Mpl, has provided a means toward understanding the molecular basis of megakaryocytic development. TPO was found to control the proliferation and differentiation of megakaryocytes and their progenitors specifically (16, 17) and to participate in the activation of platelets (18). The TPO receptor is a member of the cytokine receptor superfamily and is believed to be activated through homodimerization (19, 20). The human erythrocyte receptor, TPO receptor, c-Mpl, can be maintained in culture in the presence of phorbol esters. Interestingly, in these chemically induced differentiation models, regulatory elements that participated in the temporal expression of GPIIb were identified and located at the same positions as the tissue-specific positive and negative sequences (15). However, the identity of the factors physiologically important in the regulated expression of GPIIb in vivo awaits further investigation.

### MATERIALS AND METHODS

**Antibodies and Reagents**—Rabbit polyclonal anti-EF-1, anti-Ets-1/2, anti-GATA-1, and anti-NF-E2 antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Antibodies directed against the amino-terminal domain of PU.1/Spi-1 or against the last carboxy-terminal 50 amino acids of Fli-1 were kindly provided by Dr. F. Moreau-Gachelin (Institut Curie, Paris) and Dr. O. Delattre (Institut Curie, Paris), respectively. Rabbit polyclonal anti-Ets-1 (serum 8) and anti-GAPPs were kindly provided by Dr. J. Ghsadfel (Institut Curie, Orsay, France) and T. Sueyoshi (National Institutes of Health, Research Triangle Park, NC), respectively.

**Cell Culture and Transfections**—Human erythromegakaryoblastic UT7 cells (21) or the stable transfectant cells expressing the murine TPO receptor, UT7-Mpl (22), were cultured in a minimal essential medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) and EPO (2 units/ml, Boehringer Mannheim). The cells (5 × 10⁶/transfection) were transfected by electroporation (250 V, 960 microfarads) with 2–10 μg of the indicated luciferase reporter plasmid, 10 μg of the pCMV-gal reference plasmid, and, where indicated, 2–10 μg of expression vector. Unrelated DNAs were added to a final 50-μg amount of total foreign DNA added to the cells. The transfected cells were immediately divided into two aliquots and incubated in the basal EPO-containing culture medium at 37 °C for 24 h. The culture medium of one of the aliquots was supplemented with murine TPO or the basal EPO-containing culture medium at 37 °C.

**Western Blot Analysis**—Whole cell extracts (100 μg) or nuclear extracts (50 μg) were analyzed by electrophoresis on 10% polyacrylamide gels followed by Western blotting and immunodetection using peroxidase-coupled antibodies and chemiluminescence detection (ECL, Amer sham Corp.) as described (29).

**Electrophoretic Mobility Shift Assays (EMSAs)**—Nuclear extracts were prepared according to Schreiber et al. (28). Protein concentration was evaluated using the BCA protein assay reagent (Pierce). Double-stranded oligonucleotides were labeled with [γ-32P]ATP using T4 poly nucleotide kinase (29). Equal aliquots (2–β μg) were incubated at 4 °C for 15 min in binding buffer (10 mm HEPES (pH 8), 100 μM EDTA, 50 mM KCl, 5 mM MgCl₂, 4 mM spermidine, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin (Fraction V), 2.5% glycerol, and 4% Ficoll 400) in the absence or presence of a 100-fold excess of unlabeled oligonucleotides or 1–2 μl of the indicated antibodies. Radiolabeled probe (20,000 cpm, 0.1–0.2 ng) was then added, and the incubation was allowed to proceed for an additional 45 min. Extracts were analyzed on a 6% polyacrylamide gel in 0.5 × Tris borate running buffer as described (29). The sequences of the oligonucleotides used as probes in EMSAs were as follows: E74 (30), TCGGGCTCGAGATATACACCAAACCTCACCT- GCTC; Sp1, ATTCGATCCGGGCGGCGGCCA; GATA (bp 189 to 167 of the mouse α-globin promoter), CGGCCAACGTGAGGAT- TCCCT; G514 (bp 525 to 500 of the hGPIIb promoter), TCTCTGAAGGGAGGTCG; G463 (bp 490 to 456 of the hGPIIb promoter), CAGCTTTATCCGCGGCGGACA; G139 (bp 159 to 118 of the hGPIIb promoter), TGGGTGTCCTACCACTTCCTCTGAC- AC TCTGACCACATGA; and G39 (bp 48 to 24 of the hGPIIb promoter), AAAAGACTTCTGTTGGAGAATCTGA. The known consensus binding sites for Ets, Sp1, and GATA factors are indicated in boldface. Mutant nucleotides in known consensus binding sites are underlined.

**RESULTS**

**Thrombopoietin Activates the GPIIb Promoter in UT7-Mpl Cells**—The human UT7-Mpl cell line expressing the murine TPO receptor, c-Mpl, can be maintained in culture in the presence of GM-CSF or EPO with similar proliferative rates (22). If UT7-Mpl cells were initially maintained in the presence of EPO, the megakaryocytic differentiation induced by TPO was far greater than if cells were initially maintained in the presence of GM-CSF, with a stronger induction of the CD41 marker and the appearance of polyploid cells, as previously reported (22). The induced expression of GPIIb mRNA was correlated with an accumulation of GPIIb mRNA (22), suggesting that TPO modulated the expression of the GPIIb gene at the transcriptional level. To test this hypothesis, we introduced into UT7-Mpl cells a reporter construct composed of a fragment of the human GPIIb gene promoter (bp −787 to +33) fused to the luciferase reporter gene, and we analyzed its expression in the
Our reference plasmid (pCMV-gal) or luciferase reporter construct was introduced into UT7-Mpl cells along with the pCMV-gal reference plasmid, and cells were maintained for 48 h in culture following transfection in the presence of the indicated concentrations of TPO. Luciferase activities were evaluated and normalized relative to the β-galactosidase activity of the cotransfected reference plasmid. Normalized luciferase activities measured in TPO-treated cells were compared with activities in untreated cells (set as 1) and are expressed as -fold activation. Results are the means of three independent experiments. 

![Graph A](image1)

**Graph A.** Activation of the GPIIb promoter in TPO-treated UT7-Mpl cells. The −787/+33 hGPIIb promoter-luciferase construct was introduced into UT7-Mpl cells along with the pCMV-gal reference plasmid, and cells were maintained for 48 h in culture following transfection in the presence of the indicated concentrations of TPO. Luciferase activities were evaluated and normalized relative to the β-galactosidase activity of the cotransfected reference plasmid. Normalized luciferase activities measured in TPO-treated cells were compared with activities in untreated cells (set as 1) and are expressed as -fold activation. Results are the means of three independent experiments.

![Graph B](image2)

**Graph B.** Time course of luciferase induction after TPO treatment. Some increased expression of luciferase activity (2–3-fold) was observed after the first 6–18 h of TPO treatment (Fig. 1A). This time course of expression persisted with GM-CSF (2.5 ng/ml; white bars) or GM-CSF (2.5 ng/ml; white bars) for the times indicated. The reporter activity was measured and normalized as described for A. The activity of the control β-actin-reporter construct in TPO-treated cells is also shown (black bars).

TPO treatment stimulated luciferase activity in a dose-dependent manner, with a linear increase in the luciferase signal at TPO concentrations from 100 to 1000 units/ml (Fig. 1A). This last concentration was chosen for all subsequent experiments. The expression of phGPIIb-luc was also a function of time of TPO treatment (Fig. 1B): a moderate induction of luciferase activity (2–3-fold) was observed after the first 6–18 h of treatment, followed by a strong increase thereafter (24–48 h, 20-fold induction over untreated cells). This time course of expression of phGPIIb-luc correlated well with the kinetics of accumulation of endogenous GPIIb mRNA in TPO-treated UT7-Mpl cells (data not shown). Some increased expression of our reference plasmid (pCMV-gal) or luciferase reporter constructs under the control of the ubiquitous β-actin promoter was also detected (Fig. 1B, black bars). These findings suggest that TPO may also activate basal transcription or may possess some general stabilizing post-transcriptional effects in addition to a specific action on GPIIb promoter activity.

To test the specificity of TPO action on phGPIIb-luc expression, we also incubated transiently transfected UT7-Mpl cells with GM-CSF (Fig. 1B, white bars), interleukin-3, interleukin-6, or interferon-γ instead of or in combination with TPO. None of these cytokines affected the expression of phGPIIb-luc, and none modulated the action of TPO during the time of the transient transfection assay (data not shown), despite the expression of functional receptors for these factors in the cells (21, 29). These data indicate that the region from bp −787 to +33 of the hGPIIb gene contains a functional promoter and regulatory elements that mediate TPO-dependent transcriptional activation.

The hGPIIb Promoter Contains a TPO-responsive Enhancer That Includes an Important Ets-binding Site—To further delineate the specific DNA elements required for TPO induction, we tested deletion constructs in the region from bp −787 to +33 of the hGPIIb promoter as depicted in Fig. 2. The basal expression of this series of hGPIIb promoter-luciferase deletion constructs was first tested in the absence of TPO. In contrast to our data obtained with HEL cells (14), progressive 5′-deletions of the hGPIIb promoter down to position −307 did not result in any significant change in luciferase expression in TPO-un-treated cells (Fig. 2, left column). The next further truncations (to positions −170, −126, and −109) generated promoter fragments with the same change in transcriptional activity as we (14) and others (15) obtained in HEL and K562 cells.

Upon TPO treatment (Fig. 2, right column), we observed that deletion of the distal promoter region from bp −787 to −553 of the hGPIIb promoter did not affect the levels of TPO-induced luciferase activity. However, an additional deletion from bp −553 to −414 reproducibly decreased (−2.5-fold) the cytokine-mediated expression of the reporter gene, indicating that this 130-bp region contains a TPO-responsive element. No further deletions significantly impaired TPO-induced lucifere activity. The same results were obtained in the parental UT7 cells, where hGPIIb-reporter constructs were introduced along with a c-Mpl expression vector in similar transient transfection assays (data not shown), further strengthening our data.

The TPO-responsive region (bp −553 to −414) overlaps with a previously characterized tissue-specific megakaryocytic enhancer whose activity depends on the integrity of two motifs, namely a GATA-binding site (GATA −463) and an Ets-binding site (EBS −514) (11). To determine which of these two elements is necessary for TPO action, we tested reporter constructs containing individual mutations of these two sites in the context of the fully responsive −597/+33 hGPIIb-reporter construct. As shown in Fig. 2 (lower constructs), mutation of GATA −463 did not affect the TPO responsiveness of the hGPIIb promoter. In contrast, mutation of the sole EBS −514 (changing the Ets-binding core sequence GGAA to TTAA) resulted in a significant decrease in the TPO response, of the same magnitude as the one observed with the enhancer-deleted −414 construct (Fig. 2, upper constructs).

The hGPIIb promoter fragment (bp −787 to +33) also contains two other proximal EBSs at positions −39 and −139 from the transcriptional start site (see scheme in Fig. 2), which exhibits the known consensus binding site G(C/A)(G/A)GG/AAT of Ets family proteins (30). Surprisingly, mutations of either of these two proximal EBSs did not affect the TPO-mediated activation of the hGPIIb promoter (Fig. 2, lower constructs). However, as expected from previous studies with HEL cells (8), mutation of EBS −39 decreased the basal promoter activity of...
the hGPIIb-luc construct in TPO-untreated UT7 cells. Thus, EBS −514 is the only EBS necessary for TPO-induced expression of the hGPIIb promoter.

**TPO Induces the Binding of PU.1/Spi-1 to the Enhancer EBS**—To determine if specific regulatory proteins bind to the TPO-inducible enhancer, EMSAs were performed using oligonucleotides spanning the different EBSs of the hGPIIb promoter or an optimized EBS from the *Drosophila* E74 promoter (30). As shown in Fig. 3, the E74 oligonucleotide formed four major DNA-protein complexes (complexes C1–C4) in the presence of nuclear extracts from untreated cells (lane 1). Three of these complexes (C1, C2, and C4) were highly specific as they were inhibited by a 100-fold excess of unlabeled E74 DNA (lane 2), but not by a mutant oligonucleotide (E74mut) in which the GGAA core sequence required for Ets binding was replaced by CCAA (lane 3). Similarly, using an oligonucleotide spanning EBS −514 as a probe (the G514 oligonucleotide), four DNA-protein complexes were detected, in the presence of untreated cell extracts, that migrated as those detected in the presence of the E74 probe. However, complex C1 was often poorly detected. These complexes were all specific for Ets-binding consensus sequence as they were inhibited by an excess of unlabeled E74 DNA, but not by the E74mut oligonucleotide (lanes 7 and 8). TPO treatment of UT7-Mpl cells led to a strong increase in the quantity of the faster migrating DNA-protein complex (C4), with no detectable change in the other complexes (lanes 4–6).

This increase was initially detected after 18 h of TPO treatment (data not shown). Similar data were obtained when TPO-treated cell extracts were incubated in the presence of the E74 probe instead of G514 (Fig. 4, lanes 5 and 6). The identity of the Ets-binding protein present in this TPO-induced complex was next studied. Using a series of antibodies directed against specific Ets family members, we found that polyclonal anti-PU.1/Spi-1 antibodies totally eliminated complex C4, but failed to affect any of the other G514-protein complexes in supershift assays (Fig. 3, lane 9). The anti-Ets-1/2, anti-Fli-1, anti-GABPa (data not shown), and anti-Elf-1 (Fig. 3, lane 10) antibodies did not suppress the formation of complex C4.

We next focused our attention on the two other GPIIb EBSs (see Fig. 2). Using the same extracts as above and the oligonucleotide spanning EBS −39 or EBS −139, we never detected DNA-protein complexes migrating with mobility close to that of complex C4 or interacting with anti-PU.1/Spi-1 antibodies (data not shown). Taken together, these data demonstrate that the Ets transcription factor PU.1/Spi-1 specifically binds to EBS −514 of the GPIIb promoter (as well as to the optimized E74 EBS) and exhibits an inducible DNA binding activity following TPO treatment.

**The TPO-responsive Enhancer EBS Interacts Preferentially with PU.1/Spi-1**—To better understand the function of the TPO-responsive EBS −514, we wondered whether this EBS...
PU.1/Spi-1 Is Involved in TPO-dependent GPIIb Gene Activation

In contrast, the optimized E74 EBS interacted strongly with all three Ets factors tested (lanes 9–11).

Using the same COS cell extracts, we found that neither EBS −39 nor EBS −139 bound the PU.1/Spi-1 factor. However, both EBS −39 and EBS −139 interacted with Ets-1, although with a rather weak affinity as compared with the optimized E74 EBS (data not shown). Therefore, among the three GPIIb EBSs present in the active GPIIb promoter fragment tested, only EBS −514 specifically bound PU.1/Spi-1. These data strengthen the relevance and specificity of PU.1/Spi-1 binding to the enhancer EBS −514.

TPO Induces the Expression of PU.1/Spi-1 Protein—We further studied the expression of a number of Ets family proteins by Western blot analysis using the same UT7-Mpl extracts as those used in EMSAs (Fig. 6). We detected low levels of PU.1/Spi-1 and Fli-1, but no Ets-1, in untreated cells. TPO treatment greatly increased the level of PU.1/Spi-1 protein, which reached the same levels as those expressed in erythroleukemia HEL cells (Fig. 6A, upper panel). TPO treatment also resulted in a weak increase in the amount of Fli-1 proteins as compared with the amount in HEL cells (Fig. 6A, center panel). In contrast, Ets-1 protein was still undetectable in TPO-treated UT7-Mpl cells, although it was clearly detected in HEL cells (Fig. 6A, lower panel). Elf-1 and GABPα Ets family proteins were also expressed in UT7-Mpl extracts, but their levels were not affected by TPO treatment (data not shown).

UT7-Mpl cells were also treated with GM-CSF instead of TPO, and the expression of Ets factors was similarly assayed by Western blot analysis. As shown in Fig. 6B, even after 4 days of treatment, GM-CSF did not significantly enhance the expression of any Ets factors tested in UT7-Mpl cells. All together, our data indicate that TPO selectively up-regulated the expression of PU.1/Spi-1.

PU.1/Spi-1 Enhances the Activity of the hGPIIb Promoter—Because TPO increased the expression of PU.1/Spi-1 and Fli-1, we analyzed the effect of overexpression of PU.1/Spi-1 or Fli-1 protein on the activity of the hGPIIb promoter. The phGPIIb-luc reporter construct was cotransfected in UT7-Mpl cells along with vector expressing either PU.1/Spi-1 or Fli-1. As indicated in Fig. 7 (black bars), overexpression of either the PU.1/Spi-1 or Fli-1 factor stimulated the expression of phGPIIb-luc up to 3–4-fold in untreated cells. This increase was specific since overexpression of the PU.1/Spi-1 or Fli-1 factor did not affect the expression of an EPO receptor-luciferase reporter construct devoid of any known EBSs in similar cotransfection assays.
More important, deletion of the PU.1/Spi-1-binding site EBS−514 from the phGPIIb-reporter construct abolished the positive effect of PU.1/Spi-1 overexpression on pGPIIb-luc expression (Fig. 7, (−477) hGPIIb-luc, black bars). Although TPO alone strongly increased the expression of pGPIIb-luc, overexpression of PU.1/Spi-1 resulted in an additional increase in luciferase activity (Fig. 7, hatched bars). No such effect was found in TPO-treated cells expressing the −477 GPIIb construct, lacking the PU.1/Spi-1-specific EBS−514. In contrast, overexpression of Fli-1 attenuated the TPO-mediated activation of the hGPIIb promoter (Fig. 7), likely due to competition between Fli-1 and endogenous PU.1/Spi-1 for EBS−514.

We also introduced a dominant-negative variant of Ets proteins carrying only the DNA-binding Ets domain of Ets-1 (pDNEts) into UT7-Mpl cells. This sole Ets domain was previously reported to behave as a general dominant-negative form for all Ets family factors (26). Coexpression of the Ets domain (pDNEts) with pGPIIb-luc profoundly inhibited the TPO-dependent activation of the hGPIIb promoter while affecting only slightly the basal expression of GPIIb-luc in TPO-untreated cells (Fig. 7). The effect of this dominant-negative form of Ets proteins was specific for the expression of the GPIIb construct and was not related to a toxic side effect since it did not affect the expression of an EPO receptor-luciferase construct (data not shown). These data strengthen the importance of Ets factors in the TPO-induced expression of the GPIIb gene.

**DISCUSSION**

In this study, we provided evidence that, in the UT7-Mpl cell line, the transcription factor PU.1/Spi-1 regulates the expression of the megakaryocytic GPIIb gene through binding to a specific TPO-responsive element. To our knowledge, this is the first evidence of a direct involvement of the PU.1/Spi-1 transcription factor in the control of megakaryocytic gene expression. We further showed that TPO regulates the expression of active PU.1/Spi-1 protein in UT7-Mpl cells. This is the first evidence of the regulation of a specific Ets family transcription factor by a cytokine.

The PU.1/Spi-1 factor is an Ets-related transcription factor that was first discovered as a target of insertional activation at the Spi site by the Friend virus (31). This insertion leads to an overexpression of normal PU.1/Spi-1 proteins in infected erythroid progenitors and consequently to immortalization of erythroblasts via as yet uncharacterized mechanisms (32). Independently, PU.1/Spi-1 was identified as a macrophage and B lymphoid transcription factor (33) that regulated the expression of a number of genes in this two lineages (reviewed in Ref. 35). The expression of PU.1/Spi-1 was shown to be restricted to hematopoietic organs and was detected in myeloid, erythroid, and B (but not T) lymphoid cell lines. In bone marrow, *in situ* immunohistochemical analysis showed that PU.1/Spi-1 was present in early (but not late) erythroid and granulocytic precursors. In the same studies, PU.1/Spi-1 was shown to be surprisingly highly expressed in mature megakaryocytes (34). A number of other studies reported the expression of PU.1/Spi-1 in cultures of early erythroid progenitors (32, 35, 36), which suggested a requirement for the PU.1/Spi-1 factor during very primitive erythroid development (37). Interestingly, Friend murine erythroleukemia cells, which were derived from Friend virus-infected erythroid progenitors and overexpressed
megakaryocytes suggested that factors bound to the proximal EBS and Sp1 sites of the rat GPIIb promoter could interact with as yet uncharacterized enhancer factor(s) to form a transcription complex with the TFIID factor, joining the basal transcriptional machinery to upstream enhancer elements (40). PU.1/Spi-1 has been previously reported to directly interact via its N-terminal transactivation domain with TFIID in vitro (41). According to these observations and our present data, PU.1/Spi-1 bound to EBS -514 may provide a physical link between the basal transcription complex (TFIID) at the transcriptional start site and the enhancer region, which will regulate the magnitude of GPIIb transcription. Since a number of other known megakaryocytic enhancer genes (PF4, GPV, etc.) possess multiple Ets-binding sites in their regulatory region (3, 44), it remains to be established whether the PU.1/Spi-1 factor will also participate in the regulation of other megakaryocytic gene expression.

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