Transcriptional Regulation of Tristetraprolin by Transforming Growth Factor-β in Human T Cells*

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Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that plays a critical role in modulating immune response and inflammation. We employed the Affymetrix cDNA microarray system to detect genes whose expression is regulated by TGF-β1 in a human T cell line HuT78. Tristetraprolin (TTP), a protein involved in the degradation of tumor necrosis factor-α (TNF-α) mRNA, was found to be up-regulated by TGF-β. This up-regulation was confirmed by reverse transcriptase-PCR analysis that revealed a rapid and transient induction of TTP mRNA by TGF-β1 in HuT78 cells, primary human T cells, and THP-1 macrophage-monocyte cells. In addition, de novo protein synthesis was not required for this induction, suggesting that TTP is regulated by TGF-β at the transcriptional level. To delineate the transcriptional regulation of the TTP gene, a 2.7-kb human TTP promoter region (~2682 to +56 bp relative to the transcription initiation site) was isolated. We found that this promoter was stimulated by TGF-β1 or a constitutively active TGF-β type I receptor via TGF-β-specific Smad proteins. Furthermore, a series of TTP promoter deletion constructs were used to localize the Smad-responsive region to the −583 to −263 bp portion of the promoter. In this region, the TTP promoter contained a stretch of putative Smad-binding elements that had a synergistic effect in mediating Smad activation of the promoter. These putative Smad-binding element-containing sequences were also able to bind Smad3 and Smad4 proteins purified in vitro. As TGF-β- and TTP-deficient mice exhibit overlapping phenotypes manifested by multifocal inflammation and autoimmunity, our findings that TTP transcription is under the control of TGF-β signaling would indicate a potential role of TTP in mediating the immune suppressive action of TGF-β in vivo.

Transforming growth factor-β (TGF-β) is a multifunctional immune modulator that plays an essential role maintaining immune response and homeostasis of the immune system (1). TGF-β coordinates events critical to the progression and resolution of autoimmune-mediated inflammatory responses. This complex coordination involves recruitment of inflammatory cells, activation of lymphocytes, modulation of macrophage function, and expression of adhesion molecules by endothelial cells, monocytes, and lymphocytes. TGF-β affects a wide array of processes involved in T cell function including proliferation, apoptosis, antigen-presenting cell function, and differentiation (2). The immune suppressive function of TGF-β is elegantly illustrated by the in vivo studies in which TGF-β1 or Smad3, one of the critical proteins downstream of TGF-β receptors, are genetically disrupted in mouse. Targeted disruption of TGF-β1 or Smad3 in mice results in a severe and multifocal inflammatory response in all the pups that are born alive (3–5).

TGF-β exerts its biological effects by interacting with two transmembrane receptors, type I and type II, with serine/threonine kinase domains in the intracellular region (6). After ligand binding, the activated type I receptor relays the signal to Smad2 and Smad3 (pathway-specific Smads), which are activated by the kinase activity of the TGF-β type I receptor through phosphorylation at the C-terminal end (7, 8). Upon phosphorylation, the pathway-specific Smads form hetero-oligomeric complexes with Smad4, the common mediator Smad (9, 10). These complexes then migrate to the nucleus and activate gene transcription through either direct DNA binding by the Smad proteins or by their association with other sequence-specific transcription factors (11). The DNA binding ability of Smad proteins is mainly achieved by their MH1 domains, as indicated by the crystal structure of the Smad MH1 domain (12). For example, Smad3 and Smad4 have been shown to associate with a consensus palindromic motif GTCTAGAC (13). Smad proteins may also stimulate transcription by associating with other sequence-specific transcription factors. For example, Smad2 and/or Smad3, when complexed with Smad4, may associate with Xenopus FAST-1 (forkhead activin signal transducer-1) to regulate Mix.2 gene transcription or mouse FAST-2 to regulate Goosecoid promoter (14, 15). The transactivating activity of Smad proteins is achieved by their MH2 domains, which interact with two closely related transcriptional coactivators, CBP and p300, that link specific transcription factors with the basal transcriptional machinery (16–18).

Tristetraprolin (TTP, also known as TIS11, Nup475, and Goosecoid) is the prototype of a family of zinc finger proteins possessing a pair of closely spaced CCCH class zinc fingers (19–22). It is widely expressed with particularly high levels in spleen, lymph nodes, and thymus (19). Studies for the past few years; RT, reverse transcription; SBE, Smad-binding element; Smad, Smad and Mad-related protein; TNF-α, tumor necrosis factor-α; TTP, tristetraprolin; CHX, cycloheximide; GST, glutathione S-transferase.
years have indicated that TTP is able to promote the turnover of certain mRNAs containing AU-rich elements in their 3′-untranslated regions (23). The mRNA of several cytokines that are important for immune response, inflammation, and hematopoiesis, such as TNF-α, interleukin-3β, and granulocyte-macrophage colony stimulating factor contain this class of AU-rich elements (23, 24). The function of TTP in regulating immune response is clearly illustrated by gene deletion studies. TTP-deficient mice manifested inflammatory disorders characterized by cachexia, arthritis, and autoimmune reactions (25). These phenotypes could be alleviated by treatment with antibodies to TNF-α, further indicating that TTP is a physiological regulator of TNF-α at the animal level.

To further delineate the molecular mechanisms by which TGF-β regulates T cell functions, we employed a DNA microarray method to detect genes whose expression is regulated by TGF-β1 in HuT78 cells. The HuT78 cell line was originally established from a patient with mycosis fungoides and has been characterized as a model cell line for studying human T lymphocytes (26). HuT78 cells produce several cytokines typical for human Th0 cells in response to anti-CD3 and phorbol 12-myristate 13-acetate stimulation, such as interleukin-4 and interferon-γ (27). As a result of the microarray analysis, we found that TTP was up-regulated by TGF-β treatment. To understand the mechanism underlying the regulation of TTP expression by TGF-β, we characterized the promoter of TTP and identified the Smad-responsive region in the promoter. These studies have indicated that TTP is a transcriptional target regulated by TGF-β signaling in T cells.

**EXPERIMENTAL PROCEDURES**

**Reagents—**RPMI 1640 medium, fetal bovine serum, and penicillin/streptomycin were purchased from BioWhittaker (Walkersville, MD). Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Recombinant human activin A was provided by the National Hormone and Pituitary Program (NHP, Rockville, MD). Cytochalasin D (CHX), and actinomycin-D were purchased from Sigma.

**Cell Culture, Transient Transfection, and Enzyme-linked Immunosorbent Assay—**HuT78 and THP-1 cells were cultured at 5 × 10^5 cells/ml in RPMI 1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated (56 °C, 30 min) fetal bovine serum. Cells were seeded either in 12-well-plates (Greiner, Lake Mary, FL) at 2 ml/well or 100-mm diameter dish (Greiner) at 10 ml/dish. Human embryonic kidney 293 (HEK293) cells were cultured at 1.5 × 10^5 cells/ml in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum supplemented with penicillin and streptomycin. Cells were seeded in 24-well plates (Greiner) at 0.5 ml/well. For the culture of the primary human T cells, peripheral blood was obtained from the local blood center (Central Indiana Regional Blood Center). Buffy coats collected from whole blood by centrifugation were mixed with an equal volume of phosphate-buffered saline. The mixture was layered over Ficol-Paque Plus (Amerham Biosciences), and cells at the interface were collected. T cells were purified by negative selection with the use of a combination of monoclonal antibodies and complement (Lympho-Kwik; One Lambda, Canoga Park, CA). The T cells (1 × 10^6 cells/ml) were then activated in RPMI 1640 medium containing 10% fetal bovine serum and 50 μM β-mercaptoethanol with anti-CD3 and anti-CD28 for 3 days at concentrations of 1 and 0.5 μg/ml, respectively. The purity of the resulting activated T cells exceeded 90%. Transient cell transfection was performed by a SuperFect reagent (Qiagen, Valencia, CA) for HuT78 cells and the calcium phosphate method for HEK293 cells. The TGF-β1 level in the THP-1 culture medium was determined by an enzyme-linked immunosorbent assay kit following the enclosed protocol (BioSource International, Inc.).

**Microarray Analysis—**HuT78 cells were cultured in a 100-mm diameter dish in the presence or absence of TGF-β1 (1.25 ng/ml) for 90 min. Total RNA from these cells was isolated using RNeasy (Qiagen). Preparation of cRNA, hybridization, and scanning of the human U95A arrays were performed according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). The arrays were scanned at 3 mm with the GeneArray scanner (Affymetrix). The chips of Human Genome U95Av2 array were used and they represent about 12,000 sequences previously characterized in terms of function or disease association.

**RNA Isolation and cDNA Synthesis—**RNA from HuT78 cells was isolated using RNeasy (Qiagen, Valencia, CA). One μg of the recovered RNA was treated with RNase-free DNase I (Invitrogen, Rockville, MD) to remove the residual DNA, and reverse transcribed in a 25-μl volume reaction with oligo(dT) primer using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) to generate first strand cDNA. The products were diluted in a final volume of 1000 μl. Diluted cDNA was stored at −80 °C until used for PCR.

**PCR**—The oligonucleotides used for PCR reactions to detect the expression of TTP, Smad7, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table I. PCR reactions were performed in a total volume of 10 μl containing 10 μM Tris-HCl, 50 mM KCl, 1.0–2.5 mM MgCl₂, 0.2 μM each dNTP, 1 μM each primer, 1.0 unit of TaqDNA polymerase (Promega, Madison, WI), and 2 μl of previously diluted reverse transcription reaction. The thermal cycling parameters consisted of denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s for 20 to 35 cycles. The PCR products were separated on 2% agarose gels in 0.5 TAE and visualized with ethidium bromide (EtBr).

**Competitive RT-PCR**—Competitive RT-PCR was performed as described previously with minor modifications (28). Competitor template for the human GAPDH gene (284 bp) was synthesized by overlap extension PCR of native PCR product (566 bp) and cloned into the pBluescript vector. The deletion mutant was then amplified by PCR with the original primers and the resultant competitor template was purified by GenElute PCR DNA Purification Kit (Sigma) and the concentration was determined by absorbance at 260 nm. A constant amount of competitor template was co-amplified with reverse-transcribed samples or varying amounts of the target cDNA standard using the specific primers. Following amplification, the competitor and the target products were separated on a 2% agarose gel in TAE buffer and visualized with EtBr. The band intensity was quantified using densitometric analysis by the NIH Image program. The log ratios of the amplified competitor and the target were plotted against the initial target in a standard curve. The amount of the mRNA in samples was deduced from the equation of the linear regression of this plot.

**Plasmid and GST Fusion Proteins**—Different lengths of human TTP transcriptional promoter were PCR-amplified and subcloned into pGEX-3b luciferase vector (Promega). Deletion mutant constructs were generated by overlap-extend extension PCR followed by subcloning into pGEX-3b. The Smad expression plasmids and the constitutively active TGF-β type I receptor plasmid have been described previously (29, 30). The C-terminal truncated (ΔMH2) GST fusion proteins of Smad3 and Smad4 were generated by in-frame fusion of the N-terminal region of Smad DNA with pGEX-4T2 (Amerham Biosciences) as previously described (31). The constructs were transformed into Escherichia coli BL21 strain (Amerham Biosciences), and the GST fusion proteins were purified according to the manufacturer’s protocol.

**TABLE I**

| Transcript | Primer | Location | Sequence (5′ to 3′) | Product (bp) |
|------------|--------|----------|--------------------|-------------|
| GAPDH      | 5′     | 301–322  | GTCTCACCACCATGGAGAAGG  |
|            | 3′     | 854–866  | TCGCTTTGAACTGACAGAGGA  |
| Smad7      | 5′     | 531–552  | GTTTGCTGTGAACTTACGGG  |
|            | 3′     | 1181–1202| TCCAAAGCTGTATCGACCGG  |
| TTP        | 5′     | 491–510  | TCATCCACAAACCTAGCGA   |
|            | 3′     | 947–966  | GATGCAGGTGAAGATGGGGA  |
**Promoter Assay**—HuT78 cells or HEK293 cells were transfected with different combinations of plasmid DNA. A Renilla luciferase vector, phRL-SV40, was cotransfected to serve as an internal control for transfection efficiency. The cells were harvested at 36 h after transfection by lysis with 200 μl of TNEN lysis buffer. In TGF-β-treated groups, cells were treated with TGF-β1 (1.25 ng/ml) for 6 h before harvest. Ten microliters of the lysate was used for the dual luciferase assay (Promega). The samples were counted for 10 s in a FL12 luminometer (Zylux), and the data were represented as the relative light units.

**Gel Mobility Shift Assay**—Double stranded oligonucleotides were labeled with [32P]ATP by T4 polynucleotide kinase. The probes (about 5 x 10^6 cpm) were incubated with about 0.2 μg of GST or GST-Smad fusion proteins in a buffer containing a final concentration of 4% glyceral, 10 mM Tris (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.1 μg/μl poly(dI-dC). The nuclear extracts were prepared from HuT78 cells as previously described by others (32). The reaction was incubated on ice for 1 h and then separated by 4% nondenaturing polyacrylamide gels in 0.5 M Tris-HCl, 10 mM Tris (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.1 μg/μl poly(dI-dC). The nuclear extracts were prepared from HuT78 cells as previously described by others (32). The reaction was incubated on ice for 1 h and then separated by 4% nondenaturing polyacrylamide gels in 0.5 M Tris-HCl, 10 mM Tris (pH 7.5), 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, and 0.1 μg/μl poly(dI-dC). The nuclear extracts were prepared from HuT78 cells as previously described by others (32).

**RESULTS**

**TGF-β Augments TTP mRNA Level in HuT78 Cells in cDNA Microarray Assay**—To explore the molecular mechanisms by which TGF-β regulates T cells, we used a cDNA microarray method to analyze genes regulated by TGF-β in HuT78 cells. To catch the “immediate” response by TGF-β treatment, these cells were treated with TGF-β1 (1.25 ng/ml) for 90 min and the isolated total RNA preparation was applied to microarray analysis. The Human Genome U95Av2 array chips from Affymetrix were used and these chips contain about 12,000 sequences previously characterized in terms of function or disease association. To assure the accuracy of the data, four individual sample preparations were used for either control or TGF-β-treated cells. The relative expression level of these ~12,000 genes was detected and used in statistical analysis (t test) to reveal genes significantly changed by TGF-β. We found that a total of 107 genes are significantly altered by TGF-β, with 75 of them being increased and 32 of them decreased (Fig. 1A). Interestingly, about 27% genes up-regulated by TGF-β are involved in transcriptional regulation, indicating that these transcription factors may relay, propagate, and amplify the biological functions of TGF-β through subsequent transcriptional control. In addition, about 23% of the genes regulated by TGF-β are involved in signal transduction pathways that regulate a variety of biological functions in lymphocytes. The up-regulated genes include those previously identified to be transcriptional targets of TGF-β signaling, such as JunB (33), plasminogen activator inhibitor 1 (34), and Smad7 (30) (Fig. 1B). Interestingly, the expression level of TTP was also significantly elevated by TGF-β treatment in these cells (Fig. 1B).

**Rapid and Transient Induction of TTP by TGF-β1**—To further evaluate the effect of TGF-β and activin on TTP expression in HuT78 cells, we used RT-PCR to analyze the change in the TTP mRNA level. HuT78 cells were treated with TGF-β1 (1.25 ng/ml) or activin (20 ng/ml) and total RNA was isolated at different time points. The reverse-transcribed cDNA was first subjected to quantitative competitive RT-PCR analysis of the housekeeping gene GAPDH to determine the relative mRNA amount. A representative standard curve from competitive PCR for GAPDH is shown in Fig. 2A, in which a constant amount of competitor was co-amplified with varying concentrations of GAPDH cDNA and the intensity of the band corresponding to the competitor was decreased with increasing GAPDH cDNA (Fig. 2A, upper panel). The plot of competitor to target ratio against concentration of GAPDH cDNA exhibited a linear relationship (Fig. 2A, lower panel). No significant difference was found in GAPDH mRNA levels among all RT samples (Fig. 2B), indicating that the relative amounts of RNA were consistent among those samples. Next, changes in TTP mRNA levels in response to TGF-β and activin treatment were analyzed by RT-PCR using specific primers. Activin is another member of the TGF-β superfamily and plays an important role in regulating follicle stimulating hormone secretion in anterior pituitary (35). Activin shares several overlapping biological activities with TGF-β, partly because of the fact that both of them utilize the same Smad proteins in signaling transduction (36). As shown in Fig. 2C, GAPDH and Smad7 were also measured as an internal standard and a positive control, respectively. The intensities of the PCR products of Smad7 and TTP were normalized to GAPDH levels. The expression of TTP was increased by either TGF-β or activin and exhibited a similar time course as that of Smad7, peaking at 1 to 2 h after stimulation. Therefore, this experiment clearly indicated that TGF-β and activin were able to induce a fast and transient induction of the TTP mRNA level in HuT78 cells.

To determine whether or not TGF-β is able to induce TTP expression in other cell types, we used RT-PCR to analyze TTP mRNA levels in primary human T and THP-1 cells, a human macrophage-monocyte cell line. As shown in Fig. 3, TGF-β1 treatment in these two types of cells was able to exert a transient and rapid induction of TTP, similar to what was found in...
HuT78 cells. These data suggested that TGF-β is able to induce TTP in multiple types of cell.

**De Novo Protein Synthesis Is Not Required for TTP Induction** by TGF-β—We next investigated whether or not the induction of TTP by TGF-β requires new protein synthesis by analyzing the effect of CHX, a protein synthesis inhibitor, on the TTP mRNA level. HuT78 cells were treated with TGF-β (1.25 ng/ml) or activin A (20 ng/ml) for 2 h in the presence or absence of CHX (10 μg/ml, 1 h prior to TGF-β treatment) or D-actinomycin (D-act; 5 μg/ml, 15 min prior to TGF-β treatment). Total RNAs were isolated and subjected to competitive RT-PCR. A, GAPDH (G3PDH) mRNA levels measured by competitive RT-PCR. MeSO (DMSO) was used as a mock treatment. N and T stand for TGF-β untreated and treated, respectively. B, RT-PCR results of GAPDH, Smad7, and TTP with different PCR cycles. C, the relative levels of Smad7 and TTP as compared with that of GAPDH with 20 and 25 cycles of PCR reaction. The empty boxes and the filled boxes stand for TGF-β untreated and treated samples, respectively.
cycles for Smad7), indicating that new protein synthesis is not required for the induction of TTP and Smad7. In addition, the induction of TTP and Smad7 was diminished in the presence of actinomycin-D, suggesting that active transcription is necessary for TGF-β-mediated stimulation of these genes.

**Regulation of TTP Promoter by TGF-β and Smad Proteins**—The experiments above indicated that TGF-β and activin were able to mount a rapid and transient induction of TTP mRNA and synthesis of new proteins including transcriptional factors was not required for this induction. Activation of TGF-β/activin receptors by ligand binding exerts the downstream signaling events by phosphorylation of Smad proteins. In particular, Smad2 and Smad3 have been postulated to be the cognate Smad proteins activated by TGF-β and activin receptors and they form a complex with Smad4, followed by nuclear translocation and regulation of target genes (11). To determine whether TGF-β/activin receptor-specific Smad proteins are involved in the induction of TTP mRNA, we tested whether or not TGF-β treatment or the Smad proteins downstream of TGF-β receptor are able to transactivate TTP promoter in a reporter assay. The human TTP promoter has been previously characterized (37). Based on this information, we used PCR to isolate a 2.7-kb human TTP promoter region that spans −2682 to +56 bp relative to the transcription initiation site. This promoter region was linked to a firefly luciferase reporter and used in transient transfection experiments. HuT78 cells were transiently transfected with this reporter followed by treatment with TGF-β1. As shown in Fig. 5A (left panel), TGF-β treatment in these cells was able to significantly stimulate the TTP promoter at 6 h after the incubation. In addition, expression of a constitutively active TGF-β type I receptor in the presence of Smad3 and Smad4 was able to transactivate the promoter (Fig. 5A, right panel). These data indicated that the human TTP promoter is under the positive regulation of the TGF-β receptor activation in HuT78 cells. To further address the Smad-mediated regulation of the TTP promoter, we analyzed the promoter activity in HEK293 cells after overexpression of various Smad proteins. HEK293 cells were used because they are highly transfectable and have been successfully used to study Smad-mediated signaling (38). As shown in Fig. 5B, the 2.7-kb human TTP promoter reporter was transiently transfected in HEK293 cells. Co-transfection of Smad3, but not other Smads, was able to manifest a marked stimulation of the promoter (Fig. 5B, left panel). This is consistent with previous findings that Smad3, when overexpressed, is able to stimulate transcription of target genes in the absence of receptor activation (29). In addition, expression of Smad3 and Smad4 in the presence of a constitutively active TGF-β type I receptor was able to highly stimulate the promoter activity (Fig. 5B, right panel). These experiments provided further evidence that Smad proteins downstream of TGF-β/activin receptors were able to mediate the stimulatory effect on the TTP promoter.

**Identification of a Smad-responsive Region in the TTP Promoter**—To localize the region of TTP promoter that is transactivated by TGF-β-specific Smads, we generated a series of deletion mutants of the 2.7-kb TTP promoter. These deletion mutants were linked to a luciferase reporter and used for transient transfection of HEK293 cells. As shown in Fig. 6, co-expression of Smad3 and Smad4 was able to significantly stimulate the promoter mutants of −583/+56 bp or longer, although at a level slightly lower than that of the longest promoter, −2682/+56 bp. Interestingly, a dramatic decrease of Smad-mediated stimulation was observed with the −423/+56-bp mutants. These data indicated that the transactivating activity by Smad3 and Smad4 in the TTP promoter is mainly conferred by the sequences located within the −583 to −423-bp region. In addition, it appeared that the sequence of −2682 to −1839 bp also partially contributes to Smad-mediated activation of the TTP promoter, as the −1839/+56-bp construct had about half of the transactivating activity as that of the longest promoter.

The AGAC or GTCT repeat sequence is known to be an optimal Smad-binding element (SBE) and is found in the responsive region of several TGF-β or activin target genes including Smad7 and plasminogen activator inhibitor-1 promoters (11). Detailed sequence analysis of the TGF-β-responsive region (−583/−423 bp) of the TTP promoter revealed the presence of eight copies of GTCT repeats (Fig. 7A). In addition, an extra T in front of the GTCT repeat appears to be conserved among the TTP promoter and the majority of other TGF-β-responsive promoters previously characterized (Table II), including the promoters of JunB (33), plasminogen activator...
inhibitor-1 (34), α2(I) chain of type I collagen (COLIA2) (39), type VII collagen (40), selenoprotein (41), and Smad7 (30).

There are a total of seven copies of TGTCT in this critical region of the TTP promoter (Table II). To further characterize the possible contribution of these putative SBEs to Smad-mediated transcriptional regulation of the TTP promoter, we generated a series of mutants of the promoter with deletion of these putative SBE-containing domains. The TGF-β-responsive region of the TTP promoter was divided into three subregions based on their physical distance. These three subregions, A, B, and C, have four, two, and two copies of the GTCT repeats, respectively. We analyzed the relative contribution of these putative consensus SBEs in Smad-mediated TTP promoter activation using mutants that had each of these subregions deleted. In addition, a combination of these deletions was used in the experiment. These deletion constructs, after being linked to a luciferase reporter, were transiently transfected into HEK293 cells with the co-expression of Smad3 and Smad4.

**Fig. 6. Characterization of the TTP promoter with different deletion mutants of the promoter.** A series of deletion mutants of the human TTP promoter were cotransfected with Smad3 and Smad4 in HEK293 cells. A SV40-driven Renilla luciferase was also used to monitor the transfection efficiency. The transfected cells were lysed 36 h later and used in the dual luciferase assay. The fold change of luciferase activity induced by Smad3 and Smad4 was derived by comparing the promoter activity in the presence of Smad3 and Smad4 to that in the absence of the Smads. Data are shown as mean ± S.D.

**Fig. 7. Localization of the putative Smad-binding elements in the human TTP promoter.** A, putative SBEs of the human TTP promoter. The TTP promoter has eight copies of consensus SBEs (GTCT) and is divided into three subregions (A, B, and C). Deletion mutants of each of these subregions were generated and used in subsequent experiments. B, schematic representation of the mutation constructs and their transcriptional activities as induced by Smad3 and Smad4. HEK293 cells were transfected with various deletion constructs of the TTP promoter with empty vector or Smad3 and Smad4 expression vectors. Thirty-six hours after transfection, the cells were lysed to measure luciferase activity. -Fold induction was calculated as the ratio between the transfection with Smad3/Smad4 expression plasmids and those of empty vectors and shown as mean ± S.D. In addition, the total numbers of the GTCT repeat (4 nucleotides) or the TGTCT repeat (5 nucleotides) in each of the promoter constructs were indicated. Note that there are four GTCT repeats or one TGTCT repeat outside the −537/−396-bp region. C, -fold changes of luciferase activity at log scale were plotted against the number of putative SBEs (either GTCT or TGTCT repeats) in the reporter constructs. Note that the -fold changes are logarithmically increased with increasing number of the putative SBEs in the construct.
of the subregions were deleted, the Smad-mediated transactivation was decreased to 12–22-fold, significantly lower than the wild type promoter. Interestingly, when any two of the subregions were deleted, the fold induction by Smad3/Smad4 was further diminished to about 5–12-fold. The promoter with all three subregions deleted gave rise to a minimal level of induction by Smad3/Smad4. Furthermore, we found that the fold induction of transcription was exponentially increased with increasing numbers of the putative SBEs included in these deletion mutants. In fact, a log plot of the transcriptional fold induction versus the number of putative SBEs (either GTCT repeat or TGTCT repeat) exhibited a linear relationship (Fig. 7C). These data indicated that all these putative SBEs might be synergistically involved in Smad-mediated transactivation of the TTP promoter.

Smad3 and Smad4 Proteins Bind Directly to the Putative SBE-containing Sequence in the TTP Promoter—Our deletion studies have clearly indicated that the putative SBEs located in the /H11002 583//H11002 396-bp region are involved in transactivation of the TTP promoter by Smad protein. To provide further evidence that these putative SBE sites are implicated in Smad binding, we employed a gel mobility shift assay to determine whether or not Smad proteins could directly bind the SBE-containing sequences of the TTP promoter. The three putative SBE-containing subregions as defined above, A, B, and C (Fig. 8A), were labeled with 32P and used in a gel mobility shift assay with the C-terminal-truncated (ΔMH2) GST fusion proteins of Smad3 and Smad4. As shown in Fig. 8B, all these subregions were able to bind the purified Smad3 and Smad4 proteins, but not to the GST protein. As a positive control, we used a 33-bp SBE derived from the mouse Smad7 promoter as previously characterized by us (30). Smad3 and Smad4 exhibited comparable binding to the SBEs from the Smad7 promoter as to all of the three subregions of the human TTP promoter. Taken together, these data suggested that the putative SBEs from the critical Smad-responsive region of TTP promoter were able to physi-
cally bind the in vitro purified Smad3 and Smad4 proteins.

To further determine whether TGF-β treatment in HuT78 cells is able to induce the formation of a complex that binds the SBE-containing region of the TTP promoter, we did a similar gel mobility shift assay using nuclear extracts from TGF-β-treated HuT78 cells. In this experiment, the SBE-containing subregion C fragment described in Figs. 7 and 8 was used as the probe. As shown in Fig. 9, TGF-β treatment was able to induce a rapid complex formation that binds this subregion (Fig. 9), suggesting that Smad proteins activated by TGF-β may bind the SBE of the TTP promoter.

TGF-β Suppressed LPS-stimulated TNF-α Production in THP-1 Cells—TTP plays an important role in regulating the stability of TNF-α mRNA. This is supported by the biochemical experiments in which it was found that TTP is able to bind the AU-rich element in the 3′-untranslated region of TNF-α mRNA (23). In TTP deleted mouse, the half-life of TNF-α mRNAs are significantly prolonged (25). In this work, we found that TTP transcription is up-regulated by TGF-β. To provide a biological link between TGF-β and TNF-α regulation, we analyzed the effect of TGF-β in LPS-mediated induction of TNF-α in the human macrophage-monocyte cells, THP-1, as our study found that TGF-β is also able to induce TTP production in these cells (Fig. 3). THP-1 cells were treated with or without TGF-β1 for 4 h followed by stimulation with different amounts of LPS for 20 h. The cell culture medium was used in an enzyme-linked immunosorbent assay to determine the protein level of TNF-α. As shown in Fig. 10, LPS was able to induce TNF-α production in a dose-dependent manner. Interestingly, TGF-β treatment was able to significantly suppress the TNF-α production at different doses of LPS. These data suggested that TGF-β is able to modulate TNF-α production in these cells.

**DISCUSSION**

The present study describes that TTP is one of the immediate target genes activated by TGF-β/activin signaling in HuT78 human T cells, as revealed by both cDNA microarray analysis and RT-PCR. In addition, the human TTP was able to significantly suppress the TNF-α mRNA. This is supported by the biochemical experiments in which it was found that TTP is able to bind the AU-rich element in the 3′-untranslated region of TNF-α mRNA (23), and the half-life of TNF-α mRNAs are prolonged in TTP knockout animals (25). Taken together, these studies point to an important role for TTP in promoting the destabilization of TNF-α mRNA.

TGF-β is a multifunctional cytokine, which has been shown to modulate immune responses (1). TGF-β regulates the growth, differentiation, and function of macrophages, T cells, B cells, and NK cells. With respect to immune modulation, definitive evidence for a role in immune system regulation is derived from the studies with targeted disruption of TGF-β1 in mice. TGF-β-deficient mice exhibit a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis (3, 45). The number of circulating monocytes is elevated in these animals, and inflammatory infiltrates include large macrophages. In addition, previous studies support a role for TGF-β1 in inhibiting macrophage activation, as evidenced by the suppression of a number of activation markers including inducible nitric-oxide synthase, TNF-α, interleukin-1β, and scavenger receptor (46, 47). In the present study, we showed that TGF-β induces TTP transcription through the Smad pathway. In addition, we found that TGF-β is able to suppress TNF-α production in THP-1 cells in which TGF-β also induces TTP transcription. Because the TGF-β-deficient mice exhibit a quite similar phenotype with that of TTP-deficient mice, it is intriguing to hypothesize that the inflammatory responses in TGF-β-deficient mice may be, at least partly, caused by a decreased TTP expression. This down-regulation of TTP expression by TGF-β deficiency may consequently cause an increase in TNF-α levels, leading to the accelerated inflammation observed in the TGF-β-deficient animals. Although this issue remains to be clarified in the future, our studies here would indicate that the transcriptional regulation of TTP by TGF-β signaling may play an important role in the modulation of immune function by TGF-β.

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