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*J Immunol* 2004; 172:7263-7271; doi: 10.4049/jimmunol.172.12.7263

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The Role of mRNA Turnover in the Regulation of Tristetraprolin Expression: Evidence for an Extracellular Signal-Regulated Kinase-Specific, AU-Rich Element-Dependent, Autoregulatory Pathway

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Tristetraprolin (TTP) is a regulator of TNF-α mRNA stability and is the only trans-acting factor shown to be capable of regulating AU-rich element-dependent mRNA turnover at the level of the intact animal. Using the THP-1 myelomonocytic cell line, we demonstrated for the first time that TTP is encoded by an mRNA with a short half-life under resting conditions. Using pharmacologic inhibitors of the mitogen-activated protein kinase pathways, we show that the induction of TTP by LPS activation is mediated through changes in transcription, mRNA stability, and translation. A coordinate increase in TTP and TNF-α mRNA stability occurs within 15 min of LPS treatment, but is transduced through different mitogen-activated protein kinase pathways. This regulation of TTP and TNF-α mRNA stability is associated with the finding that TTP binds these mRNAs under both resting and LPS-activated conditions in vivo. Finally, we demonstrate that TTP can regulate reporter gene expression in a TTP 3′ untranslated region-dependent manner and identify three distinct AU-rich elements necessary to mediate this effect. Thus, TTP regulates its own expression in a manner identical to that seen with the TNF-α 3′ untranslated region, indicating that this autoregulation is mediated at the level of mRNA stability. In this manner, TTP is able to limit the production of its own proteins as well as that of TNF-α and thus limit the response of the cell to LPS. The Journal of Immunology, 2004, 172: 7263–7271.
biosynthesis in human and murine macrophages (10, 17), we predicted that this induction would be coordinately regulated at the posttranscriptional level by specific mitogen-activated protein kinase (MAPK) signaling pathways. Like TNF-α, the induction of TTP that accompanies LPS stimulation requires the presence of functionally active p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) pathways. However, substantive differences in the role of specific MAPK pathways were observed. In contrast to TNF-α mRNA, the p38 pathway played no role in the stabilization of TTP mRNA that accompanied LPS stimulation. Activation of the ERK pathway was necessary for the rapid stabilization of TTP mRNA triggered by LPS stimulation, which had no effect on TNF-α mRNA stability. Thus, while both TTF and TNF-α mRNA are stabilized by LPS stimulation, the stabilization occurs through distinct signaling pathways in the same cell. Despite these distinctions, interesting parallels in the TTP-dependent regulation of mRNA stability were discovered. Immunoprecipitation demonstrated that TTP bound to both TNF-α and TTP mRNA in vivo in both unstimulated and LPS-stimulated THP-1 cells. Furthermore, we demonstrated that TTP expression regulated reporter gene expression in a TTP 3′UTR-dependent manner, as previously observed with the TNF-α 3′UTR. The cis-acting elements through which TTP mediated its effects were localized to three functionally independent AREs clustered between nucleotides 1466–1557 in the TTP-3′UTR.

Materials and Methods

Reagents

LPS (Escherichia coli 026:B6), actinomycin D (ActD), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). SB202190 (SB), PD98059 (PD), SP600125 (JK) were purchased from Calbiochem (La Jolla, CA). GAPDH 6C5 and 5C5 Ab was produced from American Research (Beltsville, MD). Oligonucleotides were purchased from Operon Technologies (Alameda, CA).

Cell lysis and immunoblotting

Cytoplasmic preparations were prepared as previously described using a method characterized for its lack of contamination by nuclear proteins (12, 18). Cytoplasmic lysates were prepared by washing the cells twice in ice-cold PBS. All reagents and subsequent steps were used at 4 °C. The cells were lysed by gentle resuspension in 1% Triton X-100 lysis buffer (50 mM NaCl, 0.5% Triton X-100, 1 mM pefabloc, and 1 mg/ml pepstatin A). The cells were then washed with ice-cold PBS and resuspended with 0.5 vol of IP buffer and 5 μg of IP lysate were incubated in IP buffer with continuous rotation for 2 h at 4 °C first with the preculture-seum-bound beads, followed by 2 h at 4 °C with the CARP-3 Ab-bound beads. Following IP, the beads were pelleted, and washed six times with IP buffer. RNA was isolated from each immunoprecipitate by TRizol (Invitrogen, Carlsbad, CA) and then half of the isolated RNA was reverse transcribed with an oligo dT primer using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol and then analyzed by RT-PCR using the real-time PCR primers listed below.

Cell treatment and real-time PCR

THP-1 cells were treated with the indicated MAPK inhibitor or DMSO for 5 min followed by LPS (1 μg/ml) for 15 min, except DMSO alone, which was not treated with LPS. Following 15 min of LPS pretreatment ActD was added to a final concentration of 5 μg/ml at time 0. Total RNA was isolated using TRizol (Invitrogen) and polyA” mRNA was isolated using the Oligotex mRNA mini kit (Qiagen, Valencia, CA). mRNA was quantitated by spectrophotometry and diluted to 10 ng/μl. Reverse transcription was performed using Superscript II (Invitrogen), 100 ng of mRNA, and oligo dT according to the manufacturer’s protocol. For the real-time reverse transcription, the reaction was diluted 1/5 with nanopure water.

For real-time PCR, a master mix was made from the 10X SYB Green PCR Core Reagent kit (Applied Biosystems, Foster City, CA) (2.5 μl of 10X SYBR buffer, 3 μl of 25 mM MgCl2, 2 μl of dNTP Mix with dUTP, 0.125 μl of AmpliTaq Gold (5 U/μl). A primer master mix was then made using 7.625 μl of master mix, 25 pmol of each primer, and nanopure water to 20 μl). Each real-time reaction well received 20 μl of primer mix and 5 μl of sample for a final volume of 25 μl. Each experimental reaction was performed in triplicate and each loading control, serial dilution, and negative control reaction was performed in duplicate. Serial dilutions consisted of 10-fold dilutions of a previously amplified copy of the message being tested (TTP, TNF, GAPDH, actin) with four points used for each serial dilution. The four dilution points were determined empirically to cover the range of expression of the gene being tested. Negative controls included water alone as well as 100 ng of non-reverse transcribed mRNA from the zero time point or half of the immunoprecipitated RNA. Samples were run in 96-well amplification plates, with a two-step reaction 95 °C for 15 s, 64°C for 1 min for 40 cycles; each run was preceded by a 95 °C incubation for 8 min to remove the hotstart Ab from the AmpliTaq Gold and followed by a melt analysis. Primers are as follows: TNF sense, GGACACCATG AGCACTGAAAGC; TNF antisense, TGCCAGCATGCAAGAGGAG AAG; TTP sense, TCGCGACCTGGAGGCTAGT; TTP antisense, CGGCAACCTGGACACCAAC AAG; GAPDH sense, CCATCAGTGGCCAAAC CAG; GAPDH antisense,ACTTCAACGCTGTCACGT TC; PP2 antisense, CCAGCAAGGATGACGGT; PP2 antisense, TGTCAAGA AAGGTGTTAACAG.

All primer sets had efficiencies of ≥100% (±10%) and each primer efficiency was within 10% of the other primers. The correlation coefficient for each dilution series was ≥0.95. Equal loading of RNA was calculated from the actin signal using the formula (actin concentration at time X/actin concentration at time 0). The percent of remaining TTP mRNA was calculated from the actin-corrected TTP concentration using the formula [(TTP concentration time X/normalized actin signal time X)/(TTP concentration time 0/normalized actin signal time 0)] × 100, for each sample condition time point

Generation of TTP and luciferase vector constructs

Orientation of the cdNA inserts and the integrity of the DNA sequences were confirmed by sequencing using the ABI Prism Dye Terminator Cycle Sequencing kit (PerkinElmer, Wellesley, MA), and searched against the published sequence, TTP accession no. M63625, on the National Center for Biotechnology Information database using the basic local alignment search tool search program (12, 19). The cdNA insert of TTP was subcloned downstream of a CMV promoter into a pEGFP-C1 vector generated as previously described (12).

The pGL3 luciferase constructs (SV40 promoter) contain different components of the human TTP 3′UTR as follows: TTP-3′UTR, nucleotides 991–1683; TTP-991–1477, nucleotides 991–1477 lacking the ARE; TTP-1480–1684, nucleotides 1480–1684 contain the ARE; TTP- ARE, nucleotides 1466–1557, each cloned into the Xhol site in the pGL3-C 3′UTR, TTP-3′UTR and TTP-ARE fragments were generated by PCR using primers corresponding to the 20 nucleotides at each end.
of the fragment and flanked by an XbaI site. Digesting the TTP 3′UTR with XbaI and ApaI and subcloning the resulting fragments generated the TTP 991–1477 and TTP 1480–1684. Finally, TTP 3′UTR point mutant constructs were generated using the Quick-Change Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) of the TTP 3′UTR construct. These mutants change the central A to G or one of a combination of the three-pentamer sequence contained in the TTP ARE: M-1, first pentamer nucleotide 1486 A to G; M-2, second pentamer nucleotide 1501 A to G; M-3, third pentamer nucleotide 1526 A to G; M-1-2, the first and second pentamers mutated as above; M-1-3, the first and third pentamers mutated as above; M-2-3, the second and third pentamers mutated as above; TTP M-1-2-3, all three pentamers mutated as above.

**Transient transfections and luciferase assays**

HEK 293 cells were plated at a density of 300,000 cells per well in six-well plates, with RPMI 1640 supplemented with 10% FCS and 85 ng/ml gentamicin. Each well received the following: 1 µg of pGL3 luciferase vector and varying concentrations of pcDNA 3.1 His C-TTP (from 0 to 100 ng) with the difference in DNA transfected made up with the pcDNA3.1 His-C parental for a total of 1 µg in 100 µl of total, serum-free RPMI 1640 media. The DNA mixture was mixed with 4 µl of lipofectamine reagent (Life Technologies) in 100 µl of serum-free RPMI 1640, and incubated at 22°C to 25°C for 20 min following which 800 µl of serum-free media were added and the mixture placed over the cells. After 3 h, an equal volume of RPMI 1640 supplemented with 20% FCS media was added. Transfections for luciferase assays were performed in triplicate in six-well plates and each vector was used in at least three experiments. Cells were lysed 24 h after serum addition in 100 µl of 1X lysis buffer, and 20 µl of each sample was read in a luminometer according to the manufacturer’s protocol. Luciferase values were normalized for each luciferase vector with the formula [(X ng of TTP transfection/0 ng of TTP transfection) × 100].

**Statistics**

ANOVA was performed for each data set, followed by individual comparisons using a t test.

**Results**

**Regulation of LPS-induced TTP expression by the MAPK signaling pathways**

We examined the effect of LPS stimulation (1 µg/ml 2 h) on TTP protein levels in THP-1 cells. As reported, LPS treatment stimulates an increase in TTP (43 kDa) expression in THP-1 cells (10, 12–17) (Fig. 1). Previous studies of LPS induction of TTP in THP-1 cells demonstrate peak expression at 2 h (17). Similar kinetics of TTP induction were seen in monocytes and neutrophils in response to in vivo LPS administration, with peak expression at 1–2 h (17). The interaction of LPS with the Toll-like receptor 4 activates the MAPK signaling pathways, each of which play discrete roles in TNF-α biosynthesis (20). The contribution of the p38, ERK, and JNK pathways to the LPS-mediated induction of TTP and TNF-α was compared using, specifically, well-characterized pharmacologic inhibitors. THP-1 cells were preincubated with SB202190 (1 µM) (21), PD98059 (20 µM) (22), or SP600125 (20 µM).
FIGURE 3.

MAPK ACTIVITY AND TTP REGULATE TTP EXPRESSION

A

B

PD98059 (PD) 20 μM

C

SB202190 (SB) 1 μM

D

SP600125 (JK) 20 μM

E

Relative Expression

FIGURE 3.
FIGURE 4. TTP associates with TTP and TNF-α mRNA in THP-1 cells independent of LPS activation. THP-1 lysates, +/− LPS stimulation (1 µg/ml), were initially cleared with premiune serum bound to protein A-Sepharose, and subsequently TTP was immunoprecipitated. Representative data from RT-PCR (35 cycles) of control and TTP immunoprecipitates run on an agarose gel. TTP and TNF mRNA are present following TTP IP in both resting and LPS-activated THP-1 cells, while GAPDH is absent. No band is present if reverse transcriptase is not added to the reverse transcription reactions. No bands are present following the control IP. P indicates the positive control for the gene tested indicating the proper size of the band and that the primers work; one-tenth the volume of positive control is loaded, compared with experimental conditions. M indicates the 100-bp marker. All bands are of the expected 110–130 bp size.

Posttranscriptional regulation of TTP expression by LPS

The rapid rise in TTP expression following various stimuli, including LPS, a pattern associated with rapidly degraded mRNAs like TNF-α and c-Fos (24), prompted consideration that posttranscriptional mechanisms played a major role in its expression (5, 10). In addition, the TTP 3’UTR (Fig. 2) contains multiple polyuridine and ARE sequences that are associated with rapid mRNA turnover (25).

To address this, the contribution of the different MAPK pathways on TTP mRNA stability was examined. In non-LPS-stimulated THP-1 cells, TTP mRNA was highly unstable, with a half-life of 22 min (Fig. 3A). Stimulation with LPS results in a near doubling (43 min) of the TTP mRNA half-life, indicating that a component of TTP expression is regulated by mRNA stability (Fig. 3A) consistent with previous findings (2, 26). A similar effect on TNF-α mRNA stability was observed (Fig. 3A), indicating that the stability of both TTP and TNF-α mRNA is increased by LPS stimulation.

Surprisingly, when the effect of different MAPK inhibitors on TTP mRNA turnover was examined, the ERK inhibitor PD98059 (20 µM final) (Fig. 3B) reduced the stabilization of the TTP message seen with LPS activation. Indeed, PD98059 alone was sufficient to eliminate the mRNA stabilizing effect of LPS. In contrast, inhibition of the ERK pathway by PD98059 treatment had no effect on TNF-α mRNA stability (Fig. 3B). The opposite relationship was observed with inhibition of the p38 pathway. Treatment with the specific p38 inhibitor, SB202190, had no effect on TTP mRNA stability (Fig. 3C), while blunting the increase in TNF-α mRNA stability (Fig. 3C) triggered by LPS treatment.

These data indicate that LPS-mediated increases in TTP and TNF-α mRNA stability are transduced through discrete signaling pathways activated by the same stimulus in the same cell. In contrast, pharmacologic inhibition of the JNK pathway (SP600125, 20 µM final) had no effect on either TTP (Fig. 3D) or TNF-α (Fig. 3D) mRNA stability, a novel finding given the recent availability of the JNK inhibitor (23). The differing patterns of modulation of TTP and TNF-α mRNA stability seen with these inhibitors is consistent with their exerting selective and specific effects under the conditions used in these studies. In a similar vein, our observations on TNF-α mRNA turnover confirm previous work demonstrating that p38, but not ERK, inhibition alters LPS-mediated stabilization of the TNF-α message in THP-1 cells (26).

Additionally, we assessed the changes in TTP mRNA levels as a function of LPS activation, by comparing the amount of TTP message present at time 0 of the real-time PCR studies. Analysis of the data in this manner allowed us to determine the contribution of the ERK, p38, and JNK signals to changes in TTP mRNA levels triggered by LPS treatment (Fig. 3E). Because TTP is induced by TNF-α alone (10, 17), and these MAPK inhibitors can block

Table 1. Vectors to analyze TTP function on TTP 3’UTR luciferase expression

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
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<tbody>
<tr>
<td>pGL3-C</td>
<td>Vector control</td>
</tr>
<tr>
<td>pGL3-3UTR</td>
<td>TTP 3’UTR nucleotides 991–1683</td>
</tr>
<tr>
<td>pGL3-3UTR-1480–1684</td>
<td>TTP 3’UTR nucleotides 1480–1683</td>
</tr>
<tr>
<td>pGL3-3UTR-991–1477</td>
<td>TTP 3’UTR nucleotides 991–1477</td>
</tr>
<tr>
<td>pGL3-3UTR-ARE</td>
<td>TTP 3’UTR nucleotides 1466–1557</td>
</tr>
<tr>
<td>pcDNA 3.1 HIS-C</td>
<td>Mammalian expression vectors</td>
</tr>
<tr>
<td>pcDNA 3.1 HIS-C</td>
<td>Vector control</td>
</tr>
<tr>
<td>pcDNA 3.1 TTP</td>
<td>TTP expression vector nucleotides 10–1439</td>
</tr>
</tbody>
</table>

FIGURE 3. Effect of MAPK inhibitors on TNF-α mRNA stability. Following a 5-min incubation with DMSO, SB202190 (SB; 1 µM), PD98059 (PD; 20 µM), or SP600125 (SP; 20 µM), THP-1 cells were treated with LPS (1 µg/ml) for 15 min (time 0) following which ActD (5 µg/ml) was added and total cellular RNA was harvested at the indicated time points. DMSO label indicates the result from non-LPS treated cells. TTP and TNF-α mRNA levels were measured using real-time PCR and the results shown are normalized for GAPDH mRNA levels. Data shown are the mean of three experiments analyzed in triplicate with changes in mRNA levels expressed as a percentage of that seen at time 0. A, LPS stimulation stabilizes both TTP and TNF-α mRNA relative to DMSO alone at all time points (p < 0.01). B, PD98059 treatment blocks the LPS-mediated increase in TTP mRNA stability (p < 0.05), but not the stabilization of the TNF-α mRNA. C, SB202190 treatment had no effect on the LPS-mediated stabilization of the TTP message but blocked the stabilization of the TNF-α message. D, SP600125 treatment had no effect on the LPS-mediated stabilization of either TTP or TNF-α mRNA. p38 and JNK inhibition were significantly different from DMSO alone at 30 and 60 min, p < 0.03. E, Starting levels of TTP mRNA. Differential regulation of TTP expression and mRNA stability by MAPK inhibitors as indicated by the different levels of TTP mRNA at time 0. LPS and JNK show statistically significant increases (p ≤ 0.05) (+) relative to DMSO.
TNF-α biosynthesis, we minimized this potential confounding effect by exchanging changes in TTP mRNA stability after only 15 min of LPS stimulation in the absence or presence of MAPK inhibitors (Fig. 3E). LPS activation alone resulted in a 40% increase in TTP mRNA compared with unstimulated cells. This increase in TTP mRNA was abolished by p38 inhibition. In contrast, JNK inhibition had no effect on steady state levels of TTP mRNA levels, while PD98059 treatment reduced TTP mRNA levels to an intermediate degree.

**TTP binds TTP and TNF-α mRNA in vivo**

With the demonstration that TTP and TNF-α mRNA turnover were coordinately regulated by LPS activation, we examined whether TTP protein might be implicated in an autoregulatory process similar to its effects on TNF-α mRNA. We previously demonstrated that TTP interacted with the luciferase reporter mRNA in a TNF-α 3’UTR-dependent manner in vivo (293 cells transfected with 1 μg of the specified luciferase reporter constructs in the presence of various amounts of pcDNA 3.1 HIS-C or pcDNA 3.1 TTP). Data shown represent the results from at least three experiments with statistically significant (p < 0.01) dose-dependent differences between the pGL3-C control vector with plasmids containing the TTP ARE: TTP-3’UTR (nucleotides 991-1684), TTP 1480–1684, and TTP ARE (1466–1557).

**FIGURE 5.** The cis-acting elements that confer TTP responsiveness localize to an ARE-rich region in the TTP 3’UTR. 293 cells were transfected with 1 μg of the specified luciferase reporter constructs in the presence of various amounts of pcDNA 3.1 HIS-C or pcDNA 3.1 TTP. Data shown represent the results from at least three experiments with statistically significant (p < 0.01) dose-dependent differences between the pGL3-C control vector with plasmids containing the TTP ARE: TTP-3’UTR (nucleotides 991-1684), TTP 1480–1684, and TTP ARE (1466–1557).

**FIGURE 6.** TTP regulates its own expression in a 3’UTR-dependent manner through three functionally independent ARE. A, Sequence of TTP ARE point mutations (AUGUA) within ARE 1, 2, and/or 3 in the context of the entire TTP 3’UTR used in the reporter gene plasmids shown below. B, 293 cells were transfected with 1 μg of the specified luciferase reporter constructs in the presence of various amounts of pcDNA 3.1 HIS-C or pcDNA 3.1 TTP. Disruption of all three ARE, M-1-2-3, results in the loss of the ability of TTP to regulate luciferase expression. Data shown represent the results from at least three experiments with statistically significant (p < 0.01) effects on the M-1-2, M-1-3 and M-2-3 at all three TTP concentrations relative to pGL3-C. Significant differences between M-1-2-3 vs TTP 3’UTR were observed at all levels of pcDNA 3.1 TTP cotransfection (p ≤ 0.05).
distinct signaling pathways in response to the same stimulus.

Because TTP is the only identifiable ARE-dependent cis-acting element that regulates its own expression in a 3′UTR-dependent manner, similar to its regulation of TNF-α.

The role of MAPK pathways in the induction of TTP expression by LPS

Using specific pharmacologic inhibitors, we confirmed the contributions of the ERK, p38, and JNK pathways on the regulation of TNF-α mRNA expression by LPS in THP-1 cells. Inhibition of the p38 pathway blocked the rapid stabilization of TNF-α mRNA, consistent with prior studies (2, 26). In contrast, ERK and JNK inhibition had no effect on TNF-α mRNA stability (2, 26, 31). These effects were not affected by the duration (5–30) of preincubation of the inhibitor before LPS addition. The similarity of these observations to those made using both pharmacologic and molecular approaches (2, 26) indicates that these effects were attributable to their selective inhibitory effects on specific MAPK pathways.

The increase in TTP protein in response to LPS has been demonstrated in both tissue culture conditions as well as in vivo (10, 12–17). Immunoblotting indicated that, like TNF-α, each of the MAPK signaling pathways contributes to the LPS induction of TTP expression. The effect of p38 inhibition on TTP protein and mRNA has been previously reported but the mechanism was uncharacterized (10, 12–17). Despite containing a 3′UTR ARE, TTP mRNA induction by LPS was reduced by p38 inhibition without a discernable effect on mRNA turnover, suggesting a transcriptional effect, as seen with IL-1β (26, 32).

There are no previous studies examining the role of the JNK pathway on TTP expression. Pharmacologic inhibition of the JNK pathway blocked the induction of TTP by LPS without altering mRNA levels or turnover. This effect on TTP and TNF-α mRNA is identical to that seen with studies of the role of JNK activation on TNF-α biosynthesis, which we independently confirm (33, 34). These data suggest that LPS activates the JNK pathway to use translational mechanisms to enhance both TNF-α and TTP expression. Further work is required to demonstrate this conclusively.

Surprisingly, ERK inhibition by PD98059 resulted in a loss of LPS-mediated stabilization of the TTP mRNA. This is in contrast to the effect of ERK inhibition on TNF-α biosynthesis, which acts at the level of ARE-dependent nuclear export (31). In addition to TTP, the ERK pathway has been shown to regulate the stability of other mRNA including IL-6 and natural resistance-associated macrophage protein (35, 36). Our observations that both ERK and p38 pathways selectively stabilize different ARE-containing mRNAs in the same cell, in response to the same stimuli, demonstrate the complexity of ARE-dependent gene expression. This intricacy has been clearly demonstrated in mice lacking an intact p38 signaling pathway in which TNF-α and IL-6 mRNA are differentially regulated (27).

Discussion

The rapid induction of TTP by various stimuli led to its initial identification through genetic screens for early response genes (5). Because TTP is the only trans-acting factor demonstrated to regulate ARE-dependent mRNA turnover in the intact animal (10, 30), regulation of its expression is of significance. For the first time, we directly demonstrate the contribution of posttranscriptional pathways in regulating TTP expression. TTP expression is regulated in part at the level of mRNA stability, via activation of the ERK pathway. This represents a critical finding as the stability of most mRNAs was believed to be regulated by the p38 pathway. Even more significant, our data demonstrate that different messages within the same cell (TTP and TNF-α) are stabilized by distinct signaling pathways in response to the same stimulus (LPS). Adding to this complexity, we present evidence that TTP 3′UTR-dependent luciferase expression. These AREs contain TTP-independent cis-acting elements.

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Functional mapping and characterization of the interaction of TTP with its 3′UTR ARE

With the demonstration that TTP interacts with both TNF-α and TTP mRNA in vivo, we examined whether TTP might regulate its own expression. The ability of TTP to modulate reporter gene expression in a TTP 3′UTR-dependent manner localized to three AUUUUA type AREs located between nucleotides 1466–1557. Mutation of these AREs to AUGUA resulted in the loss of TTP to modulate TTP 3′UTR-dependent luciferase expression. These AREs share a core AUUUUA (UAAUUAUG, UUAUUUAUU, GUAAUUAUA) sequence. Each ARE functioned as an independent cis-acting element where TTP expression inhibited reporter gene expression. Mutation of all three ARE resulted in
loss of TTP responsiveness, despite retention of polyuridine-rich and ARE (AUUUA) sequences 5’ and 3’ to, as well as within (AUUUUA) this region.

This specificity of TTP for these ARE is consistent with prior studies in which TTP binding exhibits a clear preference for adenosine-uridine rich sequences over polyuridine sequences (37). Similarly, systematic evolution of ligands by exponential enrichment identified the nonamer UUAUUUAUU (as seen in ARE-2) as the consensus sequence, but UUAUUUAUG (ARE-1) was also seen (28). The activity of ARE-3 (GUAAUAUAUAA) as a TTP binding site would not have been predicted from these studies. This functional analysis indicates that the ability of TTP to bind and regulate ARE function is restricted to the ARE containing the sequences NUAUUUAUN, in which T, G, or A can be tolerated at the first and ninth position. This model accounts for the lack of TTP responsiveness seen with the retained AREs (AUUUUAAG, AUAAUAAGU) in the M-1-2-3 triplet molecule. This does not rule out that these ARE may function through non-TTP trans-acting factors. Rather, these data suggest a predictive model for gauging the TTP responsiveness of the numerous mRNAs identified in the ARE database (38, 39).

The independent activity of each ARE is consistent with the finding that the tandem zinc finger domain of TTP exhibits a 9-nucleotide footprint with UUAUUUAUU with two molecules capable of binding a 19-nucleotide sequence (10, 40). Because the minimum distance spanning any of the functionally active ARE in the 1466–1557 region is 24 bases, their independent regulation by TTP is consistent with the binding of an individual TTP protein. By extension, our mutational analysis indicates that TTP can bind monomerically to an ARE to influence 3’UTR-dependent reporter gene expression. This finding prompts the question of why UUAUUUAUU frequently exist in a reiterated context; is the effect of TTP on mRNA stability magnified by the binding of multiple molecules? Alternatively, does the presence of multiple ARE enhance the likelihood of TTP binding under limiting conditions or enable other trans-acting factors to bind?

**Model of TTP function**

Regulation of TTP and TNF-α mRNA stability appears to share common and discrete elements. Although TTP regulates both TNF-α and TTP expression through specific 3’UTR ARE, different signaling pathways regulate TTP and TNF-α mRNA turnover. These data suggest that the differential regulation of TTP and TNF-α mRNA stability may not always target the same cis-acting elements, consistent with the additional pathways of mRNA turnover that we have shown for TTP and has recently been shown for TNF-α (29). An alternative possibility is that the consequence of TTP binding to a specific mRNA is regulated by the phosphorylation of other proteins that interact with TTP or the mRNA (or both) to mediate mRNA decay. Thus, while TTP is phosphorylated in response to LPS activation in murine macrophages (14, 16, 41–43), these posttranslational modifications do not obviously influence its ARE-binding activity. Phosphorylation of rTTP by ERK, p38, and JNK did not alter ARE binding (14, 16, 41–43) suggesting the affinity of TTP for an ARE may not be affected by LPS activation, consistent with our in vivo binding experiments. These observations suggest that TTP may mediate both functions by serving as an “adapter” protein to regulate ARE-dependent mRNA expression. In this model, TTP binds specific AREs in a constitutive manner in macrophages, while the functional consequences of this interaction (rates of mRNA deadenylation or degradation) are determined by its interaction with other proteins which are regulated through the phosphorylation events that occur following LPS activation (44). Consistent with this model is the recent report by Stockel (45) which suggests that TTP function may be regulated through its interaction with 14-3-3 proteins. This model may explain why TTP, although present, does not regulate TNF-α mRNA stability in T cells (8). A corollary of this model is that the rapid induction of TTP mRNA and protein that accompanies macrophage activation provides cells with adequate levels of TTP to modulate the expression of the massive increase in ARE-containing mRNA.

In closing, our data indicate that in human macrophages, TTP binds to the TNF-α and TTP mRNA through their 3’UTR, leading to the ARE-dependent mRNA turnover under resting conditions. TTP and TNF-α expression are rapidly up-regulated by LPS stimulation, although the coordinate increase in their mRNA stability appears to be transduced by separate MAPK pathways. The interaction of TTP with these mRNA in vivo is not obviously affected by activation. These data suggest that LPS-dependent changes in TTP “function,” at least in terms of mediating mRNA stability, are mediated downstream to ARE binding, perhaps through interactions with other proteins that are regulated by phosphorylation. Upon removal or degradation of the activation signal, TTP shuts down its own expression as well as that of TNF-α by destabilizing the mRNA of each, contributing to the return to a resting nonactivated state.

**Acknowledgments**

We acknowledge the assistance of Alan Bergeron in the generation of Western blots for this manuscript.

**References**


