The Stability of Tristetraprolin mRNA Is Regulated by Mitogen-activated Protein Kinase p38 and by Tristetraprolin Itself*

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Tristetraprolin (TTP) is an mRNA-destabilizing protein that negatively regulates the expression of proinflammatory mediators such as tumor necrosis factor α, granulocyte/macrophage colony-stimulating factor, and cyclooxygenase 2. Here we investigate the regulation of TTP expression in the mouse macrophage cell line RAW264.7. We show that TTP mRNA is expressed in a biphasic manner following stimulation of cells with lipopolysaccharide and that the second phase of expression, like the first, is dependent on mitogen-activated protein kinase (MAPK) p38. MAPK p38 acts through a downstream kinase to stabilize TTP mRNA, and this stabilization is mediated by an adenosine/uridine-rich region at the 3′-end of the TTP 3′-untranslated region. Hence TTP is post-transcriptionally regulated in a similar manner to several proinflammatory genes. We also demonstrate that TTP is able to bind to its own 3′-untranslated region and negatively regulate its own expression, forming a feedback loop to limit expression levels.

Many of the cytokines, chemokines, and other proteins involved in the inflammatory response to infection or injury are encoded by relatively short-lived mRNAs (1, 2). These transcripts typically possess adenylate/uridylate-rich 3′-untranslated regions that contain multiple, often overlapping copies of the motif AUUUA (3–6). Such AU-rich elements (AREs) function as mRNA-destabilizing sequences that can stimulate both the deadenylation of mRNA and its subsequent degradation by the exosome, a multicompartmental 3′–5′ exonucleolytic complex (7–10). AREs contribute to transient patterns of proinflammatory gene expression by directing the rapid degradation of mRNAs following transient activation of transcription. Appropriate physiological responses to infection or injury may be dependent on this mechanism for tight regulation and constraint of cytokine expression. For example, the targeted deletion of a tumor necrosis factor α (TNFα) ARE increases the stability of murine TNFα mRNA, prolonging the expression of this cytokine in response to inflammatory stimuli and resulting in a complex inflammatory disease (11).

The half-lives of ARE-containing mRNAs are thought to be determined by their interactions with ARE-binding proteins that either promote or inhibit degradation (3–5). Several ARE-binding proteins have been described; however, clear evidence for a role in the regulation of mRNA stability exists in only a few cases. Promotion of mRNA decay is a property of the three mammalian members of the TIS11 family: TIS11 itself, also known as tristetraprolin (TTP); TIS11b, also known as butyrate response factor 1 (BRF1); and TIS11d, also known as butyrate response factor 2 (BRF2) (12–17). These proteins possess a highly conserved central domain containing two repeats of the sequence YKTELC and two unusual Cys3-His zinc fingers but are otherwise not closely related. The conserved central domain mediates interactions with ARE sequences (18) and is also present in Caenorhabditis elegans (19), Drosophila melanogaster (20), and Xenopus laevis (21) proteins. Hence the TIS11 proteins may be members of an evolutionarily ancient family of post-transcriptional regulatory factors. They can function by promoting the deadenylation of mRNAs to which they bind and/or by enhancing the recruitment of the exosome (8, 14, 16, 22).

TTP mRNA is expressed with a characteristic immediate early pattern, peaking rapidly and then returning to near basal levels within 2–4 h following stimulation of 3T3 cells with growth factors or phorbol myristate acetate (23–25), T cells with αCD3/αCD28 antibodies or transforming growth factor-β (26, 27), B cell lines with Ig cross-linking antibodies (28), and myeloid cells with lipopolysaccharide (LPS) or TNFα (13). TTP destabilizes TNFα mRNA; hence the induction of TTP gene expression by proinflammatory stimuli tends to limit the expression of TNFα (13). Mice lacking TTP display increased TNFα mRNA stability, overexpression of the cytokine, and a chronic inflammatory disease similar to that caused by deletion of the TNFα ARE (13, 29). Overexpression of granulocyte/macrophage colony-stimulating factor (GM-CSF) and overexpression of cyclooxygenase 2 (COX-2) are additional consequences of the TTP knockout (30, 31). Less direct evidence suggests that other post-transcriptional targets of TTP may include interleukin 2 (IL-2) (26), IL-3 (32), and plasminogen activator inhibitor type 2 (33). In a fibrosarcoma cell line BRF1 is essential for the destabilization of a reporter mRNA containing the IL-3 ARE (17). The members of the TIS11 family are differentially regulated in a number of cell types (25, 34). However, until knockouts of BRF1 and BRF2 are described, it will remain unclear what the physiological targets of these proteins typically possess adenylate/uridylate-rich 3′-untranslated regions that contain multiple, often overlapping copies of the motif AUUUA (3–6).
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proteins are and to what extent the functions of the TIS11 family members overlap.

It is now recognized that transient gene expression during inflammatory responses may involve not only the transient transcription of unstable mRNAs but also the dynamic regulation of mRNA stability (35). For example IL-2 mRNA is stabilized by a NFκB-dependent pathway in activated T cells (36, 37), and GM-CSF mRNA is stabilized by an ERK-dependent pathway in T24 renal cell carcinoma (38). Furthermore, the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and the phosphatidylinositol 3-kinase (PI3K) p110α pathway are negatively regulated by a PTEN-dependent mechanism (39, 40). The mechanisms underlying the regulation of unstable mRNAs are therefore complex and depend on the specific mRNA and the cellular context.

Transfection of HeLa-TO Cells—HeLa-TO cells were seeded in 6-well plates at a density of 1.5 × 10^5 cells/well. The following day cells were transfected using Superfect reagent (Qiagen). Carrier DNA (pBluescript; Stratagene) was added to keep the total amount of transfected DNA constant within experiments. 24 h after transfection, doxycycline was added to the cell medium at a final concentration of 500 ng/ml, and cells were harvested at various time points using 100 μl of Ambion lysis buffer. Lysates were passed through shredder columns (Qiagen) and stored frozen at −20 °C.

In Vitro Transcription—Template DNAs were linearized by digestion with HindIII and purified using Qiagen DNA extraction kits. Typically 1 μg of DNA template was used/reaction. Transcription reactions were performed in the presence of 1× T7 RNA polymerase buffer, 10 mM dithiothreitol, 0.5 mM each of ATP, CTP, and GTP, 10 mM MgCl2, 50 μCi of [α-32P]UTP (800 Ci mmol−1; Amersham Biosciences), and 20 units of T7 RNA polymerase (Epitector Technologies). Reactions were stopped by addition of 1 unit of RNase-free DNase I (Promega). Phenol-chloroform extraction was performed, and unincorporated nucleotides were removed using 5-200 spin columns (Amersham Biosciences) as specified by the manufacturer. Specific activities of probes were determined by scintillation counting.

Ribonuclease Protection Assay—Ribonuclease protection assays were performed using Ambion Direct Protect ribonuclease protection assay kit according to the manufacturer's instructions. Riboprobes were synthesized as described above (under “In Vitro Transcription”). Protected RNA fragments were resolved by electrophoresis on denaturing 5% polyacrylamide gels and visualized and quantified by phosphorimaging (Fuji FLA 2000).

Northern Blotting—RNA was purified from RAW264.7 cells using QIAamp RNA blood kits (Qiagen). Typically for each experimental time point between 10 and 20 μg of RNA was denatured using a mix of formamide and formaldehyde prior to loading on denaturing 4% formaldehyde gel. 18 S and 28 S ribosomal RNAs were visualized by staining with SYBR Green II (Molecular Probes) and quantified by phosphorimaging (Fuji FLA 2000). RNA samples were resolved by electrophoresis on denaturing 5% polyacrylamide gels and visualized and quantified by phosphorimaging (Fuji FLA 2000). Northern blotting was performed by standard methods, using appropriate antisera directed against p53 and cyclin D3.

Electrophoretic Mobility Shift Assay and Antibody Supershifts—RAW264.7 cell extracts were incubated with the labeled RNA probe in bandshift buffer (10 mM HEPES (pH 7.6), 3 mM MgCl2, 20 mM KCl, 1 mM dithiothreitol, 5% glycerol) for 15 min on ice in the presence of 5 μg ml−1 (final concentration) of heparin sulfate. RNA Tα, (Roche Applied Science) was then added to a final concentration of 50 units ml−1, and the reaction mixture was incubated for a further 5 min on ice. RNA-protein complexes were resolved by electrophoresis (150 V for 4 h at 4 °C) on non-denaturing 5% Tris-borate-EDTA, 4% acrylamide gel and visualized and quantified by phosphorimaging (Fuji FLA 2000). For antibody supershift experiments, the appropriate antibody was mixed with the cell extract prior to addition of the bandshift buffer, probe, and heparin sulfate, and the reaction mixture was left on ice for 30 min before addition of the RNA Tα.

Western Blotting—HeLa-TO cells were harvested by lysis in SDS-PAGE loading buffer, and lysates were run on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride. Western blotting was performed by standard methods, using appropriate antisera directed against p53 and cyclin D3.

Electrophoretic Mobility Shift Assay and Antibody Supershifts—RAW264.7 cell extracts were incubated with the labeled RNA probe in bandshift buffer (10 mM HEPES (pH 7.6), 3 mM MgCl2, 20 mM KCl, 1 mM dithiothreitol, 5% glycerol) for 15 min on ice in the presence of 5 μg ml−1 (final concentration) of heparin sulfate. RNA Tα, (Roche Applied Science) was then added to a final concentration of 50 units ml−1, and the reaction mixture was incubated for a further 5 min on ice. RNA-protein complexes were resolved by electrophoresis (150 V for 4 h at 4 °C) on non-denaturing 5% Tris-borate-EDTA, 4% acrylamide gel and visualized and quantified by phosphorimaging (Fuji FLA 2000). For antibody supershift experiments, the appropriate antibody was mixed with the cell extract prior to addition of the bandshift buffer, probe, and heparin sulfate, and the reaction mixture was left on ice for 30 min before addition of the RNA Tα.
RESULTS

LPS Stimulation of RAW264.7 Cells Induces TTP mRNA Expression in a Biphasic and p38-dependent Manner—LPS stimulation of the mouse macrophage cell line RAW264.7 caused the induction of TTP gene expression, as detected by Northern blotting, Western blotting, or electrophoretic mobility shift assays using a TNFα-3'-UTR probe (44). Expression of TTP protein remained strong 4 h after the stimulus, and the LPS-induced RNA-protein complex was detectable up to 16 h after the stimulus. This contrasted with the highly transient expression of TTP mRNA reported in several cell types, including myeloid cells (13, 27, 46). We therefore examined the time course of TTP mRNA expression in LPS-stimulated RAW264.7 cells.

In untreated cells TTP mRNA was almost undetectable (Fig. 1A). Stimulation with LPS led to a strong and rapid induction of TTP mRNA with a first peak of expression observed 1 h after the addition of the stimulus. At 2–3 h, TTP mRNA decreased toward basal level. However, TTP mRNA levels increased again at about 4 h, and a prolonged second phase of TTP mRNA expression was consistently observed. Levels of TTP mRNA during the second phase of expression ranged from 50 to 110% of those observed during the initial peak (data not shown). Like the initial peak (44), the accumulation of TTP mRNA in the second phase was significantly inhibited by 1 μM SB203580 (Fig. 1B), suggesting that both phases of gene expression are dependent upon MAPK p38.

Induction of TTP mRNA by LPS Is Independent of ERK Activation—The involvement of ERK and JNK pathways in the regulation of TTP expression was not explored previously. As described previously (47), stimulation of RAW264.7 cells with 10 ng/ml LPS resulted in a strong activation of the ERK pathway, which was almost completely inhibited by 1 μM U0126 (Fig. 2A). At this dose the induction of TTP mRNA was not significantly inhibited (Fig. 2B). A low specificity inhibitor of the JNK pathway (48, 49) did not affect the induction of TTP protein by LPS. The role of the JNK pathway in TTP gene expression was not studied further. These observations suggest a specific role for MAPK p38 in the regulation of TTP mRNA expression.

MAPK p38 Controls TTP mRNA Stability—To determine whether p38 regulated TTP expression at the level of mRNA stability, actinomycin D chase experiments were performed (Fig. 3). RAW264.7 cells were stimulated for 1 h with 10 ng/ml LPS, and transcription was then blocked by addition of actinomycin D in the presence of 1 μM SB203580 or vehicle control (0.1% Me₃SO). The decay of TTP mRNA was assessed by

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\[ \text{FIG. 1. LPS induces TTP mRNA expression in a biphasic and p38-dependent manner. A, RAW264.7 cells were either left untreated (0) or stimulated with 10 ng/ml LPS for the times indicated. TTP mRNA expression was quantified by Northern blotting and normalized for loading using 18 S rRNA. The experiment was performed twice with qualitatively identical results. B, RAW264.7 cells were stimulated with 10 ng/ml LPS for 2 h prior to the addition of either vehicle control (0.1% Me₃SO) or 1 μM SB203580. TTP mRNA levels were quantified by Northern blotting at intervals over the following 6 h and normalized against 18 S rRNA. The graph represents the mean normalized TTP mRNA levels from three independent experiments. Error bars indicate standard deviation.} \]

\[ \text{FIG. 2. Induction of TTP mRNA by LPS does not depend upon the ERK pathway. RAW 264.7 cells were incubated with either vehicle control (0.1% Me₃SO) or the mitogen-activated protein kinase inhibitor U0126 as indicated for 15 min prior to stimulation with LPS (10 ng/ml). A, cell lysates were prepared after 15 min and Western blotted for phosphorylated (activated) ERK (top panel) or total ERK (bottom panel). B, cells were harvested after 1 h, and TTP mRNA levels were quantified by Northern blotting and normalized against 18 S rRNA. The graph represents the mean normalized TTP mRNA levels from three independent experiments. Error bars indicate standard deviation.} \]

\[ \text{FIG. 3.} \]

\[ \text{A, TTP mRNA stability is regulated by MAPK p38 and TTP itself.} \]

\[ \text{B, TTP mRNA stability is regulated by MAPK p38 and TTP itself.} \]
Northern blotting over the following hour. TTP mRNA was significantly destabilized by 1 μM SB203580, demonstrating that p38 activity is required for the stabilization of the TTP transcript. Under these conditions the degradation of the TTP mRNA body was preceded by a delay of a few min; therefore, half-lives were calculated from the 10-min time point. On this basis the TTP mRNA half-life was 17 min in the absence and 6 min in the presence of 1 μM SB203580. As a negative control, 1 μM U0126 was shown to have no impact on the stability of TTP mRNA (Fig. 3, A, right-hand lanes, and B, right-hand graph).

The TTP 3′-UTR Mediates mRNA Stability by the MAPK p38 Signaling Pathway—The 3′-UTRs of cyclooxygenase 2, TNFα, IL-6, IL-8, and GM-CSF mediate mRNA stabilization by the MAPK p38 pathway; all contain multiple repeats of the RNA-destabilizing pentameric motif AUUUA that are implicated in p38 responses (42, 43, 50). The mouse TTP 3′-UTR, containing only three dispersed AUUUA motifs, is not typical of known p38-responsive sequences. To further investigate the regulation of TTP gene expression we used a doxycycline-regulated mRNA stability assay. In this system reporter mRNAs are transcribed under the control of a doxycycline-responsive promoter in HeLa-TO cells, which stably express a doxycycline-repressible transcription factor. Following addition of doxycycline to the tissue culture medium, synthesis of the reporter mRNA is rapidly and specifically switched off, and its decay can subsequently be assessed by ribonuclease protection assay. A cDNA fragment corresponding to the TTP 3′-UTR was inserted downstream of the β-globin coding region in the doxycycline-responsive reporter construct pTetBBB. The resulting construct (pTetBBB-TTP) was transiently transfected into HeLa-TO cells, with or without a vector expressing a constitutively active form of MKK6, the upstream activator of p38. 24 h after transfection, the transcription of the reporter construct was stopped by addition of 500 ng/ml doxycycline, and the cells were harvested at the indicated intervals (Fig. 4). Both chimeric β-globin-TTP and glyceraldehyde-3-phosphate dehydrogenase mRNAs were quantified by ribonuclease protection assay, the latter as an internal control for gel loading (Fig. 4). As shown in Fig. 5, a β-globin reporter mRNA (without insert) was highly stable, with little or no decay observed during a 4-h time course. Insertion of the TTP 3′-UTR led to a strong destabilization of the β-globin transcript, the chimeric reporter mRNA decaying with a half-life of 48 min (Fig. 4, A and B). In the presence of MKK6, β-globin-TTP mRNA was significantly destabilized, decaying with a half-life of 108 min. This stabilization was restored if the p38 inhibitor SB202190 was added to cells shortly before the addition of doxycycline. SB202190 did not affect the expression of MKK6 (Fig. 4C).

The MAPK p38 pathway regulates the stability of cyclooxygenase 2, IL-6, and IL-8 mRNAs via its downstream kinase MK2 (41–43). To determine whether this was also true of TTP, we coexpressed a dominant negative mutant of MK2 with β-globin-TTP mRNA. The stabilization of reporter mRNA by MKK6 was blocked by the MK2 dominant negative mutant (Fig. 4, D and E). MKK6 expression was marginally decreased by coexpression of dominant negative MK2 (Fig. 4F). However, we have found that reporter RNAs can be stabilized by only 5 ng of the MKK6 expression vector, 20-fold less than used in this experiment. Marginal variations in MKK6 expression levels are therefore extremely unlikely to be significant. In these experiments the TTP 3′-UTR behaved similarly to the cyclooxygenase 2 and IL-8 3′-UTRs despite a lack of strong structural similarity (42, 43).

A Distal AU-rich Region of the TTP 3′-UTR Mediates mRNA Stabilization by MAPK p38—The three copies of the AUUUUA motif present in the mouse TTP mRNA are concentrated within a relatively AU-rich region (~75% AU content) at the 3′-end of the transcript (Fig. 5A). To further map the TTP 3′-UTR elements required for the p38-mediated stabilization, deleted TTP 3′-UTR fragments were generated and cloned into the pTetBBB vector. The pTetBBB-TTP537 construct contains the first 537 nt, and the pTetBBB-TTP178 construct contains the last 178 nt of the TTP 3′-UTR, including all three AUUUUA motifs. Each construct was analyzed separately in the HeLa-TO reporter system. The β-globin-TTP537 transcript was stable under all conditions examined, and the addition of MKK6 had no effect (Fig. 5C). In contrast, the β-globin-TTP178 transcript was unstable but could be stabilized in the presence of MKK6 (Fig. 5D). The β-globin-TTP178 transcript was slightly more stable than the β-globin-TTP transcript (half-lives of 2 h and 48 min, respectively; Figs. 4 and 5D). However, the half-lives of both transcripts were similarly increased in the presence of MKK6. These observations suggest that the 178-nt AU-rich region contains sequences necessary for mRNA destabilization and p38-mediated stabilization. Its function may be modulated by sequences within the first 537 nt of the TTP 3′-UTR that in isolation do not possess destabilizing function.
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. GAPDH

ments as in transfected as in D. After 24 h cells were treated with vehicle control (0.1% MeSO) or 1 mM SB202190 (SB). After a further 30 min, doxycycline was added to a final concentration of 500 ng/ml. Cells were harvested at the time intervals shown, and a ribonuclease protection assay was performed to quantify β-globin-TTP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. B, mean normalized β-globin-TTP mRNA levels from three independent experiments as in A. Error bars indicate standard deviations. C, HeLa-TO cells were transfected as in A. After 24 h cells were treated with 1 mM SB202190 or vehicle control for 4 h, and then cell lysates were prepared and blotted for MKK6 and tubulin (loading control). D, HeLa-TO cells were transfected with 400 ng of pTetBBB-TTP with or without 100 ng of pCMV-MKK6EE, which expresses a constitutively active mutant of MKK6. After 24 h cells were transfected with vehicle control (0.1% MeSO) or 1 mM SB202190 (SB). After a further 30 min, doxycycline was added to a final concentration of 500 ng/ml. Cells were harvested at the time intervals shown, and ribonuclease protection assays were performed as above. E, mean normalized β-globin-TTP mRNA levels from three independent experiments as in D. Error bars indicate standard deviations. F, HeLa-TO cells were transfected as in D. After 24 h cell lysates were prepared and blotted using antibodies against the Myc epitope tag, MKK6, or tubulin.

panel, C1). An antibody that recognizes the closely related RNA-binding proteins AUF-1 and -2 generated a strong supershifted band with this probe. In the experiment shown (Fig. 6), LPS slightly decreased the intensity of the supershifted complex; however, such an effect was not reproducible. Antisera or antibodies against TTP or HuR did not supershift the 537 nt probe. The 178 nt probe containing all three AUUUA motifs generated prominent constitutive RNA-protein complexes C2 and C3, as well as an equally prominent LPS-induced complex of slightly higher mobility, C4 (Fig. 6, bottom panel). The full-length TTP 3′-UTR formed similar complexes, although their resolution was less clear (data not shown). Poor resolution of complexes is a frequent problem when relatively long probes are used in RNA electrophoretic mobility shift assays. The LPS-induced complex C4 was similar in mobility to an LPS-induced complex formed by a TNFα 3′-UTR probe, which was shown previously to contain TTP (44). Indeed C4 was strongly supershifted by an antiserum raised against the C terminus of mouse TTP, but not by the control preimmune serum (Fig. 6, bottom panel). Antibodies or antisera against AUF1/2 or HuR did not supershift either C2 or C3. All of these antibodies and antisera have been shown previously to possess supershifting activity with other RNA probes.

TTP Destabilizes a Reporter Construct Containing the TTP 3′-UTR—TTP is known to destabilize TNFα mRNA (13) and may exert this function via recruitment of the exosome (8) or via stimulation of deadenylation (16). The possibility of autoregulation of TTP biosynthesis was investigated by coexpression of TTP with a β-globin-TTP reporter mRNA. As predicted, the reporter mRNA was destabilized in the presence of exogenous TTP (Fig. 7A). TTP protein was not detectable in untransfected HeLa cells by Western blotting, whereas expression was readily detected following transfection with only 10 ng of pFLAGCMV-TTP (Fig. 7B). In the presence of MKK6 the expression of TTP was increased because of transcriptional activation of the CMV promoter. The mobility of the protein was also altered, consistent with p38-mediated phosphorylation of TTP in vivo (44). However, the apparent phosphorylation of TTP was not accompanied by a loss of destabilizing activity (Fig. 7A); in other words, destabilization of the reporter mRNA occurred both in the absence and in the presence of a constitutively active mutant of MKK6 (MKK6ca). The β-globin-TTP178 reporter mRNA was similarly destabilized by TTP, but the stability of β-globin-TTP537 RNA was not affected by coexpression of TTP (data not shown).

DISCUSSION

The mitogen-activated protein kinase p38 pathway regulates the expression of many proinflammatory genes at a post-transcriptional level by means of mRNA stabilization (39). MAPK p38 regulates the expression of TTP and may also regulate its function by means of phosphorylation (44, 51–53). This clear link between the p38 pathway and a known regulator of mRNA stability prompted us to further investigate the control of TTP gene expression by p38.

The TTP gene has been described as an immediate early

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gene that is transiently induced by mitogen and other stimuli in a number of cell types (13, 23–28, 46). We show that, in contrast, the induction of TTP mRNA in LPS-stimulated RAW264.7 cells is biphasic. Other investigators have described biphasic expression of TTP in peripheral blood leukocytes of healthy volunteers infused with a bolus dose of LPS (54). In the human monocytic cell line THP-1 the expression of TTP following an LPS challenge was again biphasic, and the second phase of expression was inhibited by TNFα neutralization (54). Although LPS-treated RAW264.7 cells abundantly secrete TNFα within 2 h of the stimulus, it is not yet clear whether the second phase of expression in these cells is TNFα-dependent. In preliminary experiments this second phase of gene expression was not impaired by a murine TNFα-neutralizing antibody. We cannot rule out the possibility that another cytokine secreted by activated RAW264.7 cells induces TTP expression in an autocrine manner. Candidates include GM-CSF and IL-6, which are secreted by macrophages and have been shown to regulate TTP expression in other cell types (55, 56).

Both the immediate and delayed phases of TTP mRNA expression in LPS-stimulated RAW264.7 cells were dependent upon the MAPK p38 pathway. In actinomycin D chase experiments TTP mRNA was strongly destabilized by 1 μM SB203580, a concentration at which p38 activity was inhibited by more than 80%, whereas the JNK pathway was scarcely affected (50). The change in TTP mRNA stability following inhibition of MAPK p38 was comparable with that of TNFα mRNA in LPS-treated RAW264.7 cells (50), COX-2 mRNA in LPS-treated human monocytes (57), or IL-6 mRNA in human IL-1β-treated fibroblast-like synoviocytes (58). As a negative control, 1 μM U0126 almost completely inhibited the activation of ERK by LPS but did not significantly inhibit the induction of TTP mRNA or alter TTP mRNA stability. We described previously an almost identical destabilization of LPS-induced TNFα mRNA by 1 μM SB203580 in RAW264.7 cells (50). Both TNFα and TTP mRNAs underwent an apparent increase in mobility following the addition of SB203580, consistent with shortening or loss of the poly(A) tail. The MAPK p38 pathway has been shown to protect ARE-containing transcripts from degradation by preventing their deadenylation (10).

To further investigate the regulation of TTP mRNA stability we used a doxycycline-regulated reporter system, which does not rely on toxic transcriptional inhibitors or kinase inhibitors of imperfect specificity. This system has provided valuable confirmation of the involvement of MAPK p38 in post-transcriptional events (42, 43, 50). In these assays the TTP 3′-UTR behaved similarly to those of COX-2, IL-6, and IL-8. It destabilized a β-globin reporter mRNA and conferred reporter mRNA stabilization by a constitutively active mutant of MKK6, an upstream activator of MAPK p38. Stabilization by MKK6 was inhibited by 1 μM SB202190 or by coexpression of a dominant negative mutant of MK2. TTP therefore appears to be post-transcriptionally regulated by the MAPK p38 pathway in a similar manner to proinflammatory gene products such as COX-2, TNFα, IL-6, and IL-8 (41–43, 50). To our knowledge this is the first study to demonstrate post-transcriptional regulation of an anti-inflammatory gene by the MAPK p38 pathway. These studies do not exclude the possibility that p38 also regulates TTP gene expression at a transcriptional level.

A large number of genes have now been shown to be post-transcriptionally regulated by the p38 pathway (39, 40). Many but by no means all of these are implicated in inflammatory or other immune responses. Those p38-responsive elements that have been mapped (11, 42, 43, 59) are invariably class II ad-
A 178-nucleotide distal region of the TTP 3′-UTR was necessary and sufficient to confer p38-reversible destabilization to a reporter mRNA. In electrophoretic mobility shift assays this region formed three complexes with cytoplasmic proteins from RAW264.7 cells. The lower mobility complexes C2 and C3 were not affected by LPS stimulation and did not contain the known ARE-binding proteins HuR or AUF1/2, consistent with an earlier report that the binding of these proteins does not correlate with p38-sensitive mRNA decay (59). The protein or proteins responsible for the formation of C2 and C3 remain to be identified. The higher mobility complex C4 was induced by LPS treatment and was shown to contain TTP itself. We have not yet mapped the residues involved in the binding of TTP; however, we note that the TTP 3′-UTR contains a single copy of the sequence UAUUUAUU, characterized previously as a preferred binding site for TTP (60).

The 537-nucleotide proximal region of the TTP 3′-UTR did not exert a destabilizing effect on its own but augmented the destabilizing effect of the distal AU-rich region. Supershifting experiments showed that the proximal region was recognized by a member or members of the AUF family of RNA-binding proteins. RAW264.7 cells express both AUF-1 and AUF-2, which appear similar in function and are both recognized by the antisera used in this experiment (61). Members of the AUF family are destabilizing factors (5, 62, 63); however, their binding may not be sufficient for destabilization of target transcripts (59). AUF proteins binding to the proximal region of the TTP 3′-UTR may cooperate with RNA-binding proteins recognizing the distal AU-rich region to regulate TTP mRNA decay.

The possibility of autoregulation of TTP expression was suggested by the presence of AUUUA motifs in the 3′-UTR and the demonstration of TTP binding to its own 3′-UTR. As predicted, a β-globin-TTP178 reporter mRNA was destabilized by TTP coexpression. The 537-nucleotide proximal region of the TTP 3′-UTR did not appear to bind TTP in electrophoretic mobility
MK2 was shown recently to phosphorylate two major sites in TTP, serines 52 and 178 (52), and these phosphorylations were suggested to result in the functional inactivation of TTP through the recruitment of 14-3-3 proteins (66). Despite strenuous efforts we have been unable to detect endogenous TTP protein in our HeLa cell lines by electrophoretic mobility shift assays or by Western blotting for example, see Fig. 7A). The antibody used detects less than 10 ng of recombinant TTP,7 and it readily detects the exogenous TTP expressed from as little as 10 ng of pFLAGCMV-TTP in transfected cells. The MAPK p38 pathway stabilizes endogenous COX-2 mRNA and exogenous reporter RNAs containing COX-2, TNFα, IL-6, IL-8, or TTP AREs in these cells (42, 43, 50, and this work). This suggests that TTP may be dispensable for p38-mediated regulation of mRNA stability, consistent with the observation that TNFα biosynthesis remains sensitive to p38 inhibitors in macrophages from a TTP knock-out mouse (67). TTP-mediated re-stabilization was correlated with TTP binding and was not a shift assays and did not mediate a response to TTP in cotransfected cells (68).

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