Tristetraprolin (TTP)-14-3-3 Complex Formation Protects TTP from Dephosphorylation by Protein Phosphatase 2a and Stabilizes Tumor Necrosis Factor-α mRNA*

Received for publication, August 2, 2006, and in revised form, December 12, 2006. Published, JBC Papers in Press, December 14, 2006, DOI 10.1074/jbc.M607347200

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Tumor necrosis factor (TNF-α) is a major cytokine produced by alveolar macrophages in response to pathogen-associated molecular patterns such as lipopolysaccharide. TNF-α secretion is regulated at both transcriptional and post-transcriptional levels. Post-transcriptional regulation occurs by modulation of TNF-α mRNA stability via the binding of tristetraprolin (TTP) to the adenosine/uridine-rich elements found in the 3′-untranslated region of the TNF-α transcript. Phosphorylation plays important roles in modulating mRNA stability, because activation of p38 MAPK by lipopolysaccharide stabilizes TNF-α mRNA. We hypothesized that the protein phosphatase 2A (PP2A) regulates this signaling pathway. Our results show that inhibition of PP2A by okadaic acid or small interference RNA significantly enhanced the stability of TNF-α mRNA. This result was associated with increased phosphorylation of p38 MAPK and MAPK-activated kinase 2 (MK-2). PP2A inhibition increased TTP phosphorylation and enhanced complex formation with chaperone protein 14-3-3. TTP physically interacted with PP2A in transfected mammalian cells. A functional consequence of TTP-14-3-3 complex formation appeared to be protection of TTP from dephosphorylation by inhibition of the binding of PP2A to phosphorylated TTP. Mutation of the MK-2 phosphorylation sites of TTP did not influence TNF-α adenosine/uridine-rich element binding and did not alter the increased TNF-α 3′-untranslated region-dependent luciferase activity induced by PP2A-small interference RNA silencing. Our data indicate that, although phosphorylation stabilizes TNF-α mRNA, PP2A regulates the mRNA stability by modulating the phosphorylation state of members of the p38/MK-2/TTP pathway.

Tumor necrosis factor-α (TNF-α)2 is a pro-inflammatory cytokine that influences a broad range of immunological pro-

*This work was supported by National Institutes of Health Grant 7R01GM066839-03 (to T. P. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: TNF, tumor necrosis factor; ARE, (adenosine/uridine)-rich element; 3′-UTR, 3′-untranslated regions; TTP, tristetraprolin; MAPK, mitogen-activated kinase; MK-2, mitogen activate protein kinase-activated kinase 2; PP2A, protein phosphatase 2A; OA, okadaic acid; LPS, lipopolysaccharide; IL-1, interleukin-1; JNK, c-Jun NH2-terminal kinase; MEK, MAPK/extracellular signal-regulated kinase kinase; GST, glutathione S-transferase; siRNA, small interference RNA.
mRNA decay. However, zinc fingers alone were not sufficient to mediate TNF-α mRNA degradation, because truncation of either the carboxyl or amino ends of TTP caused a loss TTP function (21). Both ends of the TTP zinc fingers contain a cluster of phosphorylation sites (22), yet the effects of phosphorylation on the mRNA binding and destabilizing activity of TTP remain controversial (22–25).

Phosphorylation of TTP is regulated by p38 MAPK/MK-2 pathway (22, 26, 27). Activation by MK-2 results in a complex formation with chaperone protein, 14-3-3 (22, 28). This association subsequently effects nuclear export of TTP (28, 29) and prevents TTP-associated mRNA from being targeted to the degradative machinery (30). Regulation of dephosphorylation of TTP, and its upstream pathway, is incompletely understood. PP2A is a serine-threonine protein phosphatase involved in the regulation of signal transduction, cell growth, and apoptosis (31–34). The core heterodimer of PP2A consists of a 36-kDa catalytic subunit (PP2A-C) and a 65-kDa regulatory subunit (PR65 or PP2A-A). This heterodimer interacts with a variable third subunit (PP2A-B isoforms) that influences cellular substrate specificity (35). The role of PP2A in the cellular response to inflammatory stimuli that causes cytokine expression has been described previously (30). Altered TTP-m1,2 (38) in which both zinc fingers were disrupted was kindly provided by T. Keith Blackwell (Joslin Diabetes Center, Boston, MA). GST-14-3-3 fusion protein construct and Myc-tagged 14-3-3ζ (39) was kindly provided by R. W. Holz (University of Michigan, Ann Arbor). Full-length mouse TNF-α CDNA was amplified from the total RNA of mouse lung using one-step reverse transcription-PCR (Invitrogen) and inserted into the pcDNA3.1/V5-His TOPO TA vector (Invitrogen) to obtain pcDNA3.1-TNFα-V5Hs. Control siRNA (D-001210-02) and the PP2A-siRNA (M-040657-00), PP1-siRNA (M-040960-00) targeting the catalytic subunit of mouse PP2A and PP1 were purchased from Dharmacon.

**PP2A Phosphatase Assay—**A non-radioactive, malachite green-based immunoprecipitation assay kit (Upstate Biotechnology) was used to measure PP2A activity. MHS cells were either pretreated with OA or siRNA as described. Total cellular proteins were then extracted in Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40 with no phosphatase inhibitors. Subunit C (catalytic) of PP2A was immunoprecipitated by anti-PP2A antibody. The precipitates were washed twice with lysis buffer and once with phosphatase assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂). The pellets were resuspended in assay buffer and incubated with 750 μM phosphate for 15 min at 30 °C. Reactions (25 μl) were then transferred to a microtiter plate and incubated with 100 μl of malachite green reagent. Color was developed for 5 min, and changes in absorbance were measured at 650 nm in a SpectraMAX 250 (Molecular Devices plate reader).

**Northern Blot Analysis—**Total RNA was extracted from sub-confluent 6-well plate using TRIzol reagent (Invitrogen). A total of 10 μg of RNA was resolved by 1% agarose/6% formaldehyde phosphate-buffered gel electrophoresis, blotted onto Nytran membranes using 10× SSC. Full-length 32P-labeled TNF-α antisense RNA was synthesized in vitro using plasmid pcDNA3.1-TNFα-V5His linearized with XhoI, T₇ polymerase and Maxiscript reagents and purified with Megaclear columns.
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(Ambion). The 32P-labeled β-actin probe was synthesized by the same method using the linearized plasmid provided by the Maxiscript kit. Hybridization was conducted overnight at 65 °C in hybridization solution containing 50% formamide, 5× SSC, 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyridilone, 0.2% Ficoll, 5 mM EDTA, 0.2% bovine serum albumin, and 100 μg/ml yeast RNA. After washing twice at room temperature and twice at 65 °C with 2× SSC/1% SDS, the membranes were apposed to Kodak BioMax MR film and exposed at −80 °C for 3 h.

Luciferase Assay—MHS cells were transiently transfected with pMT2-luc-UTR using Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were either pretreated with or without 1 μM OA for 1 h, washed once with cell culture medium, and then stimulated with 200 ng/ml LPS. For PP2A-siRNA silencing experiments, 0.3 μl of kinase reaction buffer containing 100 mM HEPES, pH 7.6, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 2 mM dithiothreitol, and the reaction was incubated at another 15 min. Prior to non-denaturing 6% polyacrylamide gel electrophoresis, the following antibodies were added for 15 min: 4 μg of goat anti-TTP (sc-8458, Santa Cruz Biotechnology), 1 μg of rabbit anti-14-3-3 (sc-629, Santa Cruz Biotechnology). Gels were then fixed in 12% MetOH/10% acetic acid, dried for 1 h at 60 °C, and exposed to x-ray film at −80 °C for 3–5 h.

RNA Immunoprecipitation—CO57 cells were transfected with pTet-Off, pTet-7B-ARE (TNF), and either pcDNA3 vector, pcDNA3-TTP-wt-MycHis, pcDNA3-TTP-m12-MycHis, or pcDNA3-TTP-AA-MycHis. After 24 h, cytoplasmic lysates were prepared in 1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM MgCl2, 10% glycerol, 1 mM diethiothreitol, 1 mM sodium vanadate, 50 mM NaF, 20 mM OA, 1 mg/ml heparin, and Complete protease inhibitors (Roche Applied Science). RNA was extracted from the cytoplasmic lysate using RNeagious (Ambion). MycHis-tagged TTP was immunoprecipitated using anti-Myc (9E10) antibody and protein A/G beads (Ultralink, Pierce). After eight washes with lysis buffer, RNA was isolated from the immunoprecipitated material by phenol/chloroform extraction and analyzed by Northern blot analysis as described earlier (30).

HEK293 Cell Transfection and Immunoprecipitation Assays—HEK293 cells in 35-mm dishes (0.5 × 106 cells/dish) were transfected with MycHis-tagged TTP constructs using Lipofectamine2000 (Invitrogen). Transfected cells were washed twice with ice-cold phosphate-buffered saline and lysed for 30 min at 4 °C in 600 μl of Nonidet P-40 lysis buffer containing 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche Applied Science). Co-immunoprecipitation assays were performed by incubating cell lysates with Myc-probe-agarose beads for 3 h at 4 °C. Beads were then washed twice in lysis buffer before SDS-PAGE and immunoblotting analysis.

siRNA Transfection—CO57 cells were transfected using Lipofectamine2000 (Invitrogen) with 100 nM of either a control siRNA duplex (D0) or an siRNA duplex targeting MK-2 (M2). The sequences (sense strand) were: D0, 5'-GCAUUCACUUG-GAUAGUAA-3'; M2, 5'-UCACCGAGUUAUGAACA-3' (from Ambion). After 48 h, cells were re-seeded and transfected again with the same siRNA duplexes together with either pcDNA3 (vector) or pcDNA3-TTP-wt-MycHis. Cells were cultured for an additional 48 h in serum-free medium. Where indicated, cells were treated for 30 min with 10 μg/ml anisomycin (Sigma) prior to lysis in SDS-sample buffer. HEK293 cells were transfected with control siRNA or human PP2A-siRNA using the same method as CO57 cell transfection. Control siRNA (D-001210-02) and PP2A-siRNA (L-003598-00), targeting the catalytic subunit of human PP2A, were purchased from Dharmaco.

RESULTS

PP2A Inhibition by OA or siRNA Increased LPS-induced TNF-α Secretion in MHS Cells—To investigate the role of PP2A in regulating LPS-induced TNF-α secretion from alveolar mac-
Although stimulation with 500 ng/ml LPS increased the absolute value of TNF-α secretion, the amount of TNF-α secreted into culture supernatants was nearly doubled in the OA-treated group. By 6 h of stimulation, the augmentation of TNF-α secretion was slightly less with control siRNA transfection (Fig. 2B). As expected on the basis of transfecting less confluent cell cultures with siRNA, the overall level of TNF-α secretion was significantly increased by PP2A-siRNA silencing at all time points (Fig. 2B). Of note, no increase in TNF-α secretion was observed following PP1 silencing at any time (Fig. 2B). Thus, attributing the OA-induced augmentation of TNF-α secretion to PP2A inhibition was corroborated by PP2A-siRNA silencing experiments. When feasible, siRNA was used as a complimentary strategy to confirm results from OA treatment studies.

Because recent studies suggested that PP2A positively regulates the inhibitory IκB kinase (IκB) and subsequently activates the NF-κB pathway (40), we surmised the effect of OA was largely due to post-transcriptional regulation of TNF-α mRNA. Post-transcriptional regulation of TNF-α involves the mRNA stability of its transcript. To examine the role of PP2A in the regulation of the TNF-α mRNA stability, MHS cells pretreated with...
or without OA were stimulated with LPS for 2 h and then treated with 5 μg/ml actinomycin D to block further transcription. Total RNA was extracted at the indicated time intervals. Northern blot analysis revealed that the decay of TNF-α mRNA was significantly prevented by OA pretreatment (Fig. 3). This result suggested that the effect of OA treatment on TNF-α secretion was due to the stabilization of mRNA transcript.

**PP2A Inhibition by OA or PP2A-siRNA Increased the TNF-α 3’-UTR-dependent Luciferase**—To further characterize the effects of PP2A inhibition on TNF-α mRNA stability, a luciferase reporter construct that incorporated the 3’-UTR of TNF-α (pMT2-luc-UTR) was transiently transfected into MHS cells and used to assess mRNA stability of transcribed luciferase. Transfected cells were pretreated with or without OA followed by LPS stimulation, and luciferase activity was measured at different time points. In response to OA, 2-, 15-, and 11-fold increases in luciferase activity were observed at the 2-, 4-, and 6-h time points, respectively (p < 0.01 for all time points, Fig. 4A, left panel).

Similar to OA treatment, knock-down of endogenous PP2A by siRNA also substantially increased (≈10-fold) TNF-α 3’-UTR-dependent luciferase activity at all time points tested (Fig. 4A, right panel). Because siRNA transfections were performed on less confluent cell cultures than used in OA studies, luciferase activity was normalized to micrograms of protein. As a result, no effect was seen using control siRNA as compared with non-transfected cells. Taken together, these results indicate that the unstable elements conferred by the 3’-UTR of TNF-α mRNA were suppressed by the inhibition of PP2A in MHS cells.

It has been demonstrated that activation of p38 MAPK stabilizes TNF-α mRNA (26, 27). To determine whether the effect of PP2A on TNF-α mRNA stability was mediated via the p38 pathway, inhibitors of p38, JNK, MEK1/2, and NF-κB were added 30 min after stimulation with LPS to transfected cells that had been pretreated with OA. Results showed that luciferase activity was significantly inhibited only by the p38-specific inhibitor SB 203580 (5 μM) (Fig. 4B). These data provided additional support to the hypothesis that PP2A regulated TNF-α mRNA stability via the p38 pathway.

**PP2A Inhibition by OA or PP2A-siRNA Increased Phosphorylation of p38 MAPK/MK-2**—To further support this observation, MHS cells pretreated with or without OA were

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**FIGURE 3. TNF-α mRNA stability analysis of MHS cells.** 2 × 10⁶ MHS cells were preincubated with or without 1 μM OA for 1 h at 37 °C and stimulated with 200 ng/ml LPS for 2 h. Cells were then treated with 5 μg/ml actinomycin D to block transcription and total RNA was isolated at 0.5, 1, 1.5, 2, and 3 h. Northern blot was performed as described under “Experimental Procedures.” The bottom panel shows the quantitative densitometric analysis of bands shown in the upper panel. TNF-α signals were normalized to β-actin signals, and the 0-h value was set at 100%. The Northern blot is representative of two separate experiments.

**FIGURE 4. Phosphatase inhibition by OA and PP2A-siRNA increased TNF-α 3’-UTR-dependent luciferase activity.** A, for OA treatment (left panel), MHS cells were transfected with pMT2-luc-UTR for 48 h, pretreated with or without 1 μM OA for 1 h and then stimulated with 200 ng/ml LPS. For siRNA silencing (right panel), MHS cells were first transfected with control siRNA or PP2A siRNA for 48 h and then co-transfected with pMT2-luc-UTR and siRNA for another 24 h. Cell lysates were subjected to luciferase activity analysis as described under “Experimental Procedures.” Results shown are representative of four independent experiments. B, transfected MHS cells were pretreated with or without 1 μM OA for 1 h and then stimulated with LPS for 30 min before the addition of p38, JNK, MEK1/2, and NF-κB inhibitors and further stimulated with LPS for 4 h. Triplicate wells were prepared for each sample, and the values are expressed as mean ± S.E. Results shown are representative of two independent experiments.
stimulated with 200 ng/ml LPS for the indicated time intervals. Cell lysates were assayed for phospho-p38 and its downstream kinase, phospho-MK-2, by Western blot. OA pretreatment increased the LPS-induced phosphorylation status of both p38 MAPK (Fig. 5A, upper panel) and MK-2 (Fig. 5B, upper panel). To corroborate these data with increased kinase activity, p38 phosphorylation was examined by immunoblotting the cell lysates for phospho-p38 (Fig. 5A, middle panel) and MK-2 (Fig. 5B, middle panel). As expected, OA strongly increased the LPS-induced phosphorylation status of both p38 MAPK (Fig. 5A) and MK-2 (Fig. 5B). These data further confirmed the specific involvement of PP2A in the p38 MAPK/MK-2 post-transcriptional pathway.

**PP2A Inhibition Increased Phosphorylation of TTP—RNA-binding protein TTP is a substrate for MK-2 (22, 27) and has been implicated in the 3′-UTR-mediated degradation of TNF-α (19, 20). MK-2 phosphorylates TTP at two major serine sites, Ser-52 and Ser-178, both of which serve as 14-3-3 binding sites (22, 28, 30). To measure MK-2-induced phosphorylation of TTP, a phospho-specific antibody was raised against Ser-178. The antibody recognized endogenous TTP in lysates of LPS-activated Raw264.7 macrophages (Fig. 6A, lane 2), whereas treatment of the lysate with alkaline phosphatase abolished the signal (lane 3). To confirm specificity of this antibody, COS7 cells were transiently transfected with TTP constructs. The novel, phospho-specific antibody recognized both human and mouse TTP (Fig. 6B, lanes 2 and 3) but failed to recognize TTP that was mutated at Ser-178 (lanes 5 and 6). After knocking down MK-2 in COS7 cells by siRNA, the phospho-(Ser-178)-TTP signal was reduced by ~50%, both in the absence and presence of the p38-MAPK activator anisomycin (Fig. 6C). Consistent with prior studies (22, 30), this result confirmed that MK-2 was required for phosphorylation of TTP at Ser-178.

To examine the effects of PP2A inhibition on TTP phosphorylation, MHS cells pretreated with or without OA were stimulated with LPS and then immunoblotted against phospho-serine-178 TTP (Fig. 6D). Phosphorylated TTP was pulled down by GST-14-3-3 beads in vitro and analyzed using the phospho-(Ser-178)-TTP antibody. The amount of total TTP in the cell lysates was detected with an affinity-purified TTP antibody (CARP-3). LPS-induced TTP expression with its phosphorylation being apparent by 1 h (Fig. 6D, upper panel). At 2 h, a second, lower mobility band was induced, suggesting an enhanced phosphorylation state. OA strongly
increased the phosphorylated bands, whereas incubation with recombinant PP2A significantly decreased phospho-(Ser-178)-TTP (Fig. 6D, upper panel). p38 activation is known to increase TTP expression by preventing its proteolytic degradation via the proteasome (41) or by activating its expression at the transcriptional level. Because OA treatment increased p38 activity (Fig. 5A), it was crucial to determine the total amount of TTP expression in these experiments. As expected, increased total TTP expression was seen in MHS cells after OA treatment (Fig. 6D, bottom panel). However, a substantial amount of OA-augmented TTP protein was the phosphorylated isoform, because densitometry analysis showed that total TTP expression increased ~2.5-fold after OA treatment, whereas the phosphorylated isoform (Ser-178 of TTP) increased ~4.2-fold.

FIGURE 6. Establishing specificity of phospho-(Ser-178)-specific TTP antibody. A, cells were stimulated for 4 h with LPS (10 ng/ml), and cytoplasmic lysates were prepared using RNA immunoprecipitation buffer. A portion of the lysate prepared in the absence of phosphatase inhibitors was treated for 20 min at 37 °C with 1 unit/ml alkaline phosphatase (lane 3). B, COS7 cells were transfected with human and mouse TTP cDNAs, and additional TTP constructs in which serine 52 and serine 178 were substituted by alanine. C, COS7 cells were transfected over a period of 4 days with either a control siRNA or siRNA targeting MK-2, and co-transfected with TTP. Cells were cultured for 48 h in the absence of FCS and treated with control medium or 10 μg/ml anisomycin for 30 min prior to lysis. Knock-down efficiency was monitored using an MK-2 antibody. Serine 82 of Hsp27 is a known target site of MK-2. D, phosphatase inhibition increased LPS-induced phosphorylation of TTP. GST-14-3-3 fusion proteins were expressed in E. coli HB101 cells by isopropyl-β-d-thiogalactoside induction. Bacterial lysates were incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) for 1 h at 4 °C and washed three times with phosphate-buffered saline. GST alone or GST-14-3-3 fusion protein attached to glutathione-Sepharose beads were added to the lysates and incubated for 2 h at 4 °C. Proteins pulled down by GST-14-3-3 beads were analyzed with a phospho-(Ser-178)-specific TTP antibody. Total TTP in the lysates was detected with non-phospho-TTP (CARP-3) antibody. After blotting a similar increase of PP2A binding to double Ser-pho-TTP (S52A/S178A) (Fig. 7A, first panel). To identify TTP-PP2A interaction, HEK293 cell lysates were immunoprecipitated with Myc antibody and immunoblotted for 14-3-3 (Fig. 7A, upper panel) and the catalytic subunit PP2A-C (Fig. 7A, second panel). Mutation of Ser-52 to Ala weakly reduced the assembly of TTP-14-3-3, whereas Ser-178 to Ala and Ser-52/178 to Ala-52/Ala-178 mutations substantially reduced TTP-14-3-3 association (Fig. 7A, upper panel). PP2A was also detected on the beads, suggesting that TTP was physically interacting with endogenous PP2A in vivo. Interestingly, expression of TTP-S178A showed an increased interaction with PP2A with a further increase observed with expression of the double mutation (TTP-S52A/S178A) (Fig. 7A, second panel). When reversing the order of immunoprecipitation to pull down PP2A-C first and then detect TTP using anti-Myc immuno-
siRNA in this model system increased the signal of phospho-(Ser-178)-TTP by ~50%, whether or not 14-3-3 was overexpressed (Fig. 7C, lanes 2 and 4). Also, whether PP2A was knocked down or not, the absence of 14-3-3 overexpression correlated with a diminished phospho-TTP signal (lanes 3 and 4). These data provided further evidence that TTP-14-3-3 complex formation appeared to prevent TTP dephosphorylation by PP2A.

**PP2A Interacts with and Dephosphorylates TTP from LPS-stimulated MHS Cells**—Although these data using an overexpression system suggested direct regulation of TTP phosphorylation state by PP2A, an indirect effect could not be excluded. Nor was it certain this biology could be extrapolated to immune-relevant cells such as macrophages. Therefore, to further determine whether endogenous PP2A could directly mediate TTP dephosphorylation, we first immunoprecipitated PP2A from unstimulated MHS cells. This intraperitoneal product was then incubated (30 °C, from 5 to 60 min) with cell lysates recovered from MHS cells stimulated with LPS for 4 h (Fig. 8). Lysates were then subjected to Western blot analysis for the phosphorylated and dephosphorylated isoforms of TTP on the basis of molecular weights of the protein. Dephosphorylation appeared at 15 min and longer incubation with immunoprecipitated, endogenous PP2A resulted in complete dephosphorylation of TTP (Fig. 8, upper panel). To further detect the interaction of TTP and PP2A, the beads were washed and then subjected to reducing Laemmli buffer, boiling, and immunoblotting with anti-TTP (Carp-3) antibody. As shown in Fig. 8 (middle panel), the presence of TTP on anti-PP2A-IgG-beads correlated with decreased TTP phosphorylation (lanes 4–7). As an important control, no dephosphorylation of TTP and no TTP-PP2A interaction were observed with the immunoprecipitated product using an isotype-matched, irrelevant antibody (lane 1).

**Effects of TTP Phosphorylation on TNF-α ARE Binding**—To determine the effect of TTP phosphorylation on mRNA binding, RNA gel-shift assays were performed. Cytoplasmic extracts from LPS-stimulated MHS cells were incubated...
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with a TNF-α ARE probe without the addition of supershifting antibodies (Fig. 9A). Of the five major bands resolved by non-denaturing gel electrophoresis, LPS induced one band designated in the prior literature as C4 (lanes 2 and 3) (27). Pretreatment with OA strongly increased this C4 band signal (lanes 4 and 5) by ~3.3-fold as compared with non-treated samples (lanes 2–3).

The induced C4 band was supershifted by both 14-3-3 antibody (lanes 4–8) and TTP antibody (lanes 13 and 14) (Fig. 9B), which was consistent with prior observations (30). As a negative control, no supershift was observed with an isotype-matched, control antibody (Fig. 9B, lanes 1–3 and 9–11). The increased C4 band resulting from OA treatment was similarly supershifted by anti-14-3-3 (lanes 7 and 8) and anti-TTP (lane 14) antibody. Because OA increased the amount of total TTP (see Fig. 6D) this experimental approach could not determine whether phosphorylation increased ARE-binding.

We therefore used an RNA-immunoprecipitation method to examine the ability of wild-type TTP (mTTP-wt) and Ser to Ala double mutation TTP (TTP-S52A/S178A) to co-immunoprecipitate a reporter mRNA containing the TNF-α-ARE (Fig. 9C). Globin-ARE (TNF) mRNA was effectively precipitated by both constructs with nearly identical efficiency. The TTP-m1,2 expressed possesses an altered zinc finger section of TTP such that it cannot bind TNF-α mRNA and was used as a negative control to demonstrate the specificity of this experiment (lane 3). These data suggested that TTP binding to the TNF-α ARE may be independent of the two MK-2-dependent phosphorylation sites (22, 28, 30).

Ser-52/178 → Ala-52/178 Mutation of TTP Did Not disrupt the Increased Luciferase Activity Induced by PP2A-siRNA Silencing—Because Ser-52/178 to Ala-52/178 mutation did not change the binding of TTP to TNF-α ARE, we tested the effects of this mutation on luciferase reporter gene expression and whether knocking down PP2A altered this expression. Compared with vector control, co-transfection of reporter gene (pMT2-luc-UTR) with either wild-type TTP (mTTP-wt) or TTP-S52A/S178A strongly inhibited the luciferase gene expression (data not shown). However, higher luciferase activity was observed for wild-type TTP than the mutated TTP (Fig. 10, left panel compared with right panel). This finding is consistent with prior reports of MK-2 phosphorylating TTP to stabilize TNF-α mRNA (21, 30, 42). Knock-down of PP2A increased the luciferase activity of both constructs, although less dramatically with the mutated TTP (Fig. 10). Nevertheless, it was surprising that PP2A silencing increased the luciferase activity of the mutated TTP to any degree as the two MK-2 phosphorylation sites in this construct have been mutated. It may be that the increased p38/MK-2 activity caused by PP2A silencing was responsible for the increased luciferase activity of the mutated TTP. Alternatively, other phosphorylation sites on TTP, independent of MK-2, that regulate TNF-α mRNA stability could be additional targets by PP2A.
DISCUSSION

Our laboratory has been interested in identifying the role of endogenous proteins that negatively modulate inflammatory cell signaling pathways with the hopes of identifying additional therapeutic targets. A number of inflammation-triggered disease states affecting critically ill patients (e.g. sepsis and acute respiratory distress syndrome) are initiated by pathogen-mediated activation of cells of the innate immune system. Because of this key role played by cells derived from the mononuclear/macrophage lineage, we have focused our current studies on this cell type. A canonical pathogen-associated molecular pattern that triggers cellular activation is the Gram-negative bacterial cell wall product, LPS. LPS is known to activate a myriad of pathways essential to the subsequent expression of a number of gene products. These genes include cytokines (e.g. TNF-α), chemokines, and adhesion molecules that mediate pathological responses in inflammatory disease states (43, 44). As a result, a number of studies have examined the transcriptional and post-transcriptional mechanisms regulating LPS-induced TNF-α expression.

Post-transcriptional regulation of TNF-α is mediated by binding of TTP to the AU-rich elements in the 3′-untranslated region of TNF-α mRNA. The p38 MAPK has been shown to increase TNF-α expression via ARE-mediated mRNA stability (26, 27). This action of p38 is mediated through MK-2, which phosphorylates TTP in vitro and in vivo (22, 30, 45, 46). It has been established that regulation of phosphorylated proteins results from a balance between the activity of specific kinases and phosphatases. The current studies aimed to elucidate the role of PP2A in the post-transcriptional regulation of TNF-α. We observed that modulating PP2A activity greatly enhanced LPS-induced TNF-α mRNA stability. These data provide the first evidence for the involvement of PP2A in the post-transcriptional regulation of TNF-α production. Although previous results have shown that TTP phosphorylation modulates TNF-α expression (21, 30), our understanding of the molecular mechanism by which phospho-TTP controls inflammatory gene expression remains incomplete.

Previous studies have established that binding of phosphorylated TTP to 14-3-3 (22, 28) mediates a variety of cellular functions. For example, the TTP-14-3-3 interaction was reported to be responsible for the nuclear exportation of TTP (29, 47). This observation suggests subcellular localization of the complex was an important contributor to function. This concept of localization-dependent function was supported by the additional observation that TTP-14-3-3 interaction reduced the entry of TTP into the stress granule after arsenite treatment in COS7 cells (30). In the current study, we have identified a potential additional and novel function of TTP-14-3-3 association. Our results demonstrate that interaction with 14-3-3 protects phosphorylated TTP from access by the catalytic unit of PP2A thus preventing its dephosphorylation.

We hypothesize that this protective role of TTP-14-3-3 complex formation against PP2A dephosphorylation is crucial for stabilizing cytokine messenger RNA after stimulus exposure. Our experiments suggest that LPS-induced p38 MAPK/MK-2 phosphorylation is quick and transient. Phosphorylation peaks at 30 min and is followed by subsequent, rapid dephosphorylation. In contrast, TTP phosphorylation usually persists for several hours (27, 30). Thus, it appears that 14-3-3 binding may be critical for maintaining a prolonged phosphorylation state of TTP once upstream kinases have been deactivated. Subsequently, via an unknown mechanism, 14-3-3 is dissociated, leaving the unbound TTP to be accessed by PP2A for dephosphorylation, thereby promoting mRNA decay. A recent study reported that phosphorylation of 14-3-3 by IκκB and protein kinase Cδ resulted in the dissociation of 14-3-3 from the β4GalT1 mRNA thereby enhancing mRNA stability (48). These reports support our hypothesis that dissociation of 14-3-3 from its binding to TTP might be dependent on the phosphorylation state of 14-3-3. Such a function could reflect another layer to the complexity of TTP-mediated mRNA stability.

It has been established that binding of TTP to 14-3-3 stabilizes TNF-α mRNA. This is at least in part p38/MK-2-dependent, because mutation of two key serine residues affected luciferase expression mediated by the TNF-α 3′-UTR. Importantly, in the current studies, this mutated TTP had reduced binding to 14-3-3 and showed increased association with PP2A. Thus, the enhanced mRNA decay observed with the Ser-mutated TTP appears consistent with our hypothesis. However, these results cannot exclude other phosphorylation sites as being critically involved in the stability of mRNA transcript. Consistent with our observations, recent MK-2/TTP function studies (21) as well as additional studies employing the MK-2 knockout mice (42) have suggested that alternative pathways other than p38/MK-2 could modify TTP to affect its function. An indirect effect of p38/MK-2 activation caused by PP2A inhibition could play a role in the increased stability of TNF-α mRNA; however, direct dephosphorylation of TTP by PP2A was substantiated by the present data. These two regulatory mechanisms (p38/MK-2-dependent versus independent pathways)
may not be mutually exclusive. Instead the regulatory mechanism may act at different stages of TTP activation/phosphorylation. For an example, p38 and MK-2 act immediately upon cellular activation to phosphorylate TTP and stabilize mRNA. In contrast, PP2A may function at a later stage to dephosphorylate TTP thereby affecting mRNA stability.

The current studies also examined the effect of TTP phosphorylation on its binding to mRNA. Other studies exploring this question have reported varying results (22–25). Carballo et al. reported that phosphorylated TTP bound less to a granulocyte macrophage-colony stimulating factor ARE probe than dephosphorylated TTP. This finding suggested that the increased stability of granulocyte macrophage-colony stimulating factor mRNA after LPS stimulation was caused by the release of TTP from the transcript (23). In a similar manner, Hitti et al. (42) reported that a lower phosphorylation state of TTP correlated to increased binding to a TNF-α ARE probe. In contrast to these observations, we did not detect a difference between wild-type TTP and the Ser-mutated TTP (S52A/H11032). In a similar manner, 14-3-3 binding appears to prevent the transcript to either the exosome, a multiprotein complex possessing the 5′-to-3′ exoribonucleases (50, 51), or the decapping complex possessing the 5′-to-3′ exoribonuclease Xrn1 (52, 53).

Therefore, TTP-RNA binding alone may not be the only indicator of mRNA processing. Identification of additional putative proteins involved in the ARE-binding complex requires further study. Further insight into the role of these proteins in modifying mRNA stability will be necessary. Finally, understanding how this function is influenced by their phosphorylation state and regulated by phosphatases such as PP2A is necessary to increase our understanding of the molecular regulation of cytokine gene expression.

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