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Posttranslational Regulation of Tristetraprolin Subcellular Localization and Protein Stability by p38 Mitogen-Activated Protein Kinase and Extracellular Signal-Regulated Kinase Pathways

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The p38 mitogen-activated protein kinase (MAPK) signaling pathway, acting through the downstream kinase MK2, regulates the stability of many proinflammatory mRNAs that contain adenosine/uridine-rich elements (AREs). It is thought to do this by modulating the expression or activity of ARE-binding proteins that regulate mRNA turnover. MK2 phosphorylates the ARE-binding and mRNA-destabilizing protein tristetraprolin (TTP) at serines 52 and 178. Here we show that the p38 MAPK pathway regulates the subcellular localization and stability of TTP protein. A p38 MAPK inhibitor causes rapid dephosphorylation of TTP, relocalization from the cytoplasm to the nucleus, and degradation by the 20S/26S proteasome. Hence, continuous activity of the p38 MAPK pathway is required to maintain the phosphorylation status, cytoplasmic localization, and stability of TTP protein. The regulation of both subcellular localization and protein stability is dependent on MK2 and on the integrity of serines 52 and 178. Furthermore, the extracellular signal-regulated kinase (ERK) pathway synergizes with the p38 MAPK pathway to regulate both stability and localization of TTP. This effect is independent of kinases that are known to be synergistically activated by ERK and p38 MAPK. We present a model for the actions of TTP and the p38 MAPK pathway during distinct phases of the inflammatory response.

The tandem zinc finger protein tristetraprolin (TTP; also known as Nup475, Tis11, or Zfp36) (23, 26, 40, 46, 62) is expressed in activated monocytic cells (13, 47) and T lymphocytes (49, 51). It functions to regulate the expression of tumor necrosis factor α (TNF-α) by binding to a conserved adenosine/uridine-rich element (ARE) within the 3′-untranslated region of TNF-α mRNA (13, 31, 32, 36, 47). TTP promotes both mRNA deadenylation and 3′ to 5′ degradation of the mRNA body (35, 37–39), consistent with its ability to recruit several factors involved in these processes (14, 25, 39, 45). The pivotal role of TTP in the regulation of TNF-α is illustrated by the proinflammatory phenotype of a $TTP^{-/-}$ mouse strain, in which chronic overexpression of TNF-α by macrophages results in severe polyarthritis and cachexia (11, 13, 57). TTP has also been implicated in the posttranscriptional regulation of granulocyte-macrophage colony-stimulating factor (12), interleukin-2 (51), cyclooxygenase 2 (COX-2) (50), and inducible nitric oxide synthase (24). It may also regulate its own expression by binding to an ARE in the 3′ untranslated region of TTP mRNA (60). The minimum binding site of TTP is the nonameric sequence UUAUUUAUU (2, 3, 38, 65), and it is likely that additional posttranscriptional targets of TTP containing this sequence remain to be identified.

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The p38 mitogen-activated protein kinase (MAPK) and its downstream kinase MK2 play a central role in the posttranscriptional regulation of inflammatory gene expression in myeloid and other cells (5, 16, 20–22, 33, 34, 54). We and others have therefore investigated interactions of the p38 MAPK pathway with TTP. In a mouse macrophage-like cell line, RAW 264.7, the expression of TTP was dependent on p38 MAPK, at least in part due to p38 MAPK-dependent stabilization of TTP mRNA (47, 60). TTP can be phosphorylated in vitro by p38 MAPK itself (8, 10) or by MK2 (47). Two major sites of MK2-mediated phosphorylation of mouse TTP in vitro and in vivo were identified as serines 52 and 178 (15; T. Santalucia, M. Brook, E. Hitti, G. Sully, R. Wait, C. R. Tchen, C. J. A. Asensio, M. Gaestel, J. Saklavala, and A. R. Clark, unpublished data). These phosphorylations were shown to result in the recruitment of 14-3-3 proteins, functional adaptors that specifically interact with certain serine- or threonine-phosphorylated proteins (4, 15). The recruitment of 14-3-3 proteins led to exclusion of TTP from stress granules (56), cytoplasmic structures at which translationally stalled transcripts accumulate under conditions of environmental stress (29). The phosphorylation of TTP and its exclusion from stress granules were associated with stabilization of an ARE-containing reporter mRNA (56). However, a more recent report questioned whether 14-3-3 proteins are recruited to TTP in a phosphorylation-dependent manner and whether the activation of the p38 MAPK pathway has any effect upon TTP function (52). In our hands, TTP activity was modulated but not ablated through p38 MAPK-dependent phosphorylation of serines 52 and 178 (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). In that study we also demonstrated that MK2-mediated phos-

phorylation at serines 52 and 178 promoted redistribution of TTP from the nucleus to the cytoplasm of HeLa cells.

The impact of the p38 MAPK pathway on TTP expression and function remains controversial. Several previous studies have been performed by means of transient transfection of cell types that normally express little or no TTP. In HeLa cells, the effects of the p38 MAPK pathway on subcellular localization of TTP were obscured when the protein was expressed at high levels (T. Santalucia, M. Brook, E. Hitti, et al., unpublished), while in 293 cells overexpression of TTP resulted in a paradoxical stabilization of an ARE-containing reporter mRNA (36). In lipopolysaccharide (LPS)-treated RAW 264.7 cells, as many as 10 distinct, differentially phosphorylated forms of TTP can be resolved by polyacrylamide gel electrophoresis (PAGE) and detected by Western blotting, suggesting that its posttranslational modification is highly complex (M. Brook, unpublished observations) and that additional phosphorylation events remain to be identified. Indeed, while the manuscript was in revision, Cao et al. have described over 30 putative phosphorylation sites in human TTP for which functions remain to be determined (7). In contrast, the phosphorylation of TTP in transfected HeLa cells appears much less complex (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). There is clearly a case for studying the impact of signaling pathways on the biology of endogenous rather than exogenous TTP. Where this is not possible, great care should be taken to avoid overexpression of the protein, which is likely to give rise to artifacts. With this in mind, we undertook a study of the effects of p38 MAPK and other signaling pathways on endogenous TTP in RAW 264.7 cells and primary murine or human myeloid cells, all of which express TTP when stimulated with LPS. We also optimized a transfection procedure to express exogenous TTP in myeloid cells at levels similar to those of the endogenous protein. We present evidence for complex posttranslational regulation of TTP by the extracellular signal-regulated kinase (ERK) and p38 MAPK pathways.

MATERIALS AND METHODS

Materials. All reagents were purchased from Merck Biosciences, Ltd., unless stated otherwise. *Salmonella enterica* serovar Typhimurium LPS was from Sigma-Aldrich Company, Ltd., and was used at a concentration of 10 ng/ml. The rabbit polyclonal antiserum against the N terminus of mouse TTP was described previously (47). Cycloheximide (Cx), wide-range sodium dodecyl sulfate (SDS)-PAGE molecular weight markers and a mouse monoclonal antibody against α-tubulin were from Sigma-Aldrich. SeeBlue prestained SDS-PAGE molecular weight markers were from Invitrogen. Rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) antibody was from Cell Signaling Technology, mouse anti-lamin A/C antibody was from Becton Dickson, and rabbit anti-green fluorescent protein (GFP) antibody was from Abcam. GFP-TTP expression plasmids were constructed by subcloning the previously described wild-type and S52/178A mutated murine TTP cDNAs (60) into peGFPc1 (BD Clontech). Immortalized MK2$^{-/-}$ macrophages were provided by Matthias Gaestel (Medical School, Hannover, Germany) and have been described previously (26a). MSK-1$^{-/-}$ MSK-2$^{-/-}$ mice have been described previously (55). CPG57380 was a kind gift of Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, United Kingdom).

Cell culture. RAW 264.7 cells (ATCC TIB-71) and immortalized MK2$^{-/-}$ macrophages were cultured in Dulbecco's modified Eagle's medium (Labtech) supplemented with 10% (vol/vol) fetal calf serum (PAA) in a humidified atmosphere of 5% CO_2 at 37°C.

Primary cell isolation. Elutriation of human monocytes was performed as described previously (20), and cells were cultured in RPMI 1640 supplemented with 1% (vol/vol) fetal calf serum (PAA) in a humidified atmosphere of 5% CO_2 at 37°C. Mouse bone marrow-derived macrophages were isolated as described previously (44) and were cultured in Dulbecco's modified Eagle's medium (Labtech) supplemented with 10% (vol/vol) fetal calf serum (PAA) and 10 ng/ml macrophage-colony stimulating factor (Peprotech) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection. Eighty-five percent confluent T150 flasks of macrophages were seeded into fresh T150 flasks at a ratio of 1:2 and cultured overnight in standard conditions. Prior to transfection, the cells were scraped into RPMI 1640 containing 20% (vol/vol) fetal calf serum and then pelleted at 600 × *g* for 5 min. Cells (2×10^6) were resuspended in 100 μl Nucleofection Solution T (Amaxa) and mixed with 5 μg LPS-free plasmid DNA containing 500 ng peGFPc1 and/or peGFPc1-TTP or peGFPc1-TTP S52/178A in an electroporation cuvette. The cells were electroporated using the Nucleofector device (Amaxa) program T-20 and immediately resuspended in RPMI 1640 containing 20% (vol/vol) fetal calf serum. Identical transfections were pooled to control for differences in transfection efficiency prior to seeding for culture into 6-well plates. For protein stability experiments, cells were cultured for 2 h and then either left unstimulated or treated with LPS for 2 h prior to harvest or treatment with cycloheximide and dimethyl sulfoxide (DMSO) or kinase inhibitors for a further 2 h. In some experiments, GFP expression was monitored by fluorescence microscopy or flow cytometry to quantify transfection efficiencies.

Preparation of cell extracts. All operations were performed at 0 to 4°C. The cells were rinsed twice with phosphate-buffered saline (PBS) and harvested by scraping. The cells were then pelleted by centrifugation at 600 × *g* for 10 min. Whole-cell extracts were generated by lysing the cells in 1× Laemmli sample buffer for 5 min, after which the genomic DNA was sheared using a Qiashredder column (QIAGEN) according to the manufacturer's recommendations. Cytoplasmic extracts were prepared by lysing the cells for 5 min in cytoplasmic extraction buffer (10 mM HEPES, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 50 mM sodium β-glycerophosphate, 5% [vol/vol] glycerol, 0.5% [vol/vol] Igepal CA630, 2 mM NaF, 1 mM sodium orthovanadate [all from Sigma-Aldrich]) containing 1 μM microcystin-LR (Merck Biosciences-Calbiochem), 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 10 μM E64, 1 μg/ml pepstatin A, and 10 μg/ml aprotinin (all Sigma-Aldrich). Nuclei were pelleted by centrifuging at 600 × *g* for 5 min, and the supernatant was retained as cytoplasmic extract. The nuclei were then washed by gentle pipetting in nuclei wash buffer (10 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 50 mM sodium β-glycerophosphate, 2 mM NaF, 1 mM sodium orthovanadate) containing 1 mM DTT, 1 μM microcystin-LR, 1 mM phenylmethylsulfonyl fluoride, 10 μM E64, 1 μg/ml pepstatin A, and 10 μg/ml aprotinin and pelleted by centrifuging at 600 × *g* for 5 min. The supernatant was discarded, and the nuclei were resuspended in an equal volume of nuclei wash buffer to that used for cytoplasmic extraction buffer and lysed by the addition of 4× Laemmli sample buffer to a final concentration of 1×. The genomic DNA was sheared using a Qiashredder column as per whole-cell extract production. Protein concentrations were determined by Bradford protein assay.

Western blotting. Cell extracts were prepared as described above, separated by SDS-PAGE, and electrophoretically transferred to polyvinylidene fluoride microporous membrane (Perkin Elmer). Immunoblotting was performed using standard procedures with primary antibody at either a 1:1,000 dilution or the manufacturer's recommended dilution. Horseradish peroxidase-coupled secondary antibodies (Dako) were then used according to the manufacturer's instructions, and the activity was detected using an enhanced chemiluminescence system (GE Healthcare).

Northern blotting. RNA was purified from RAW 264.7 cells using QIAamp RNA blood kits (QIAGEN). For each experimental time point, 10 μg of total RNA was subjected to denaturing electrophoresis as described previously (5). A previously described 1-kb full-length murine TTP cDNA probe (60) was labeled using ReadyToGo reagents (GE Healthcare) in the presence of 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) (GE Healthcare). Prior to transfer of the gel, 18S rRNA levels were visualized by staining with Sybr green II (Invitrogen-Molecular Probes) and quantified using a phosphorimager (FLA2000; Fuji). RNAs were capillary transferred onto a nylon membrane (Hybond XL; GE Healthcare) prior to blotting using Ultrahyb blotting solution (Ambion) according to the manufacturer's recommendations.

RNase protection assay. Assays were performed using the DirectProtect RNase protection assay kit (Ambion) according to the manufacturer's recommendations. Riboprobes complementary to murine TTP and murine TNF-α were described previously (60), and a riboprobe template (pTriGAPDH) complementary to murine GAPDH was from Ambion.

Quantitative reverse transcription-PCR. PCR was carried out as described previously (17). Briefly, TTP mRNA levels were quantified in duplicate PCRs on two independent macrophage preparations per genotype. In addition, we both confirmed the efficiency of the PCR and cloned and sequenced the PCR product

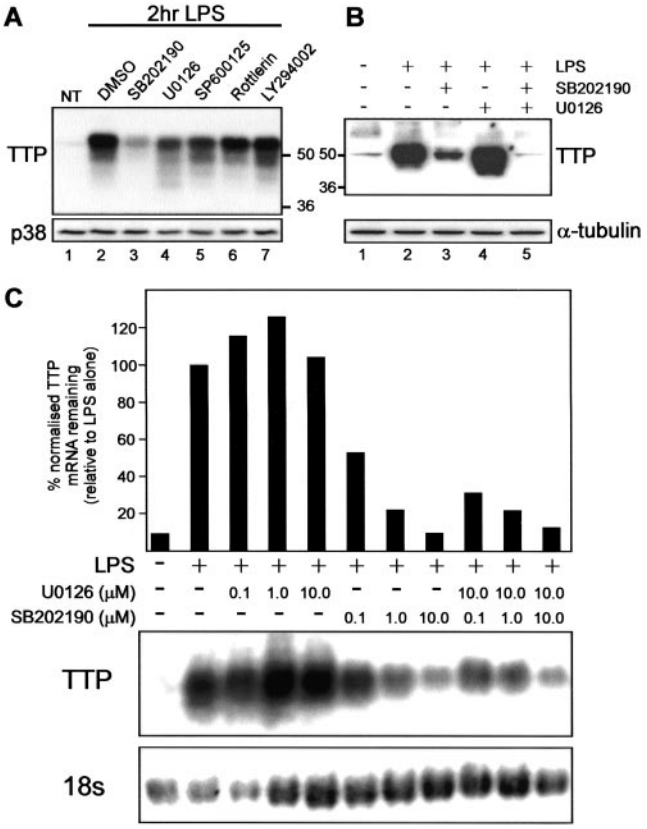

FIG. 1. TTP protein, but not mRNA expression, is synergistically regulated by the p38 MAPK and ERK pathways. (A and B) RAW 264.7 cells were either left unstimulated or stimulated with LPS for 2 h in the presence of either 0.1% DMSO, 1 μM SB202190, 10 μM U0126, 50 μM SP600125, 10 μM Rottlerin, or 10 μM LY294002. Whole-cell extracts were prepared, and TTP and tubulin were detected by Western blotting. Positions of molecular mass markers (in kilodaltons) are indicated. (C) RAW 264.7 cells were left unstimulated or stimulated with LPS for 2 h in the presence of 0.1% DMSO or the indicated concentrations of SB202190 or U0126. TTP mRNA was detected by Northern blotting and normalized against 18S rRNA. NT, untreated.

to ensure high-fidelity amplification of TTP cDNA. PCR primers were as follows: 5′, TCGGACCTACTCAGAAAGC; 3′, GGTAGAACTTGTGGCAGAG.

RESULTS

p38 MAPK and ERK signaling pathways regulate TTP expression, phosphorylation, and subcellular localization. TTP is rapidly induced in macrophages following LPS stimulation, is highly phosphorylated, and is almost exclusively cytoplasmic (9, 47). We previously demonstrated that p38 MAPK controls the expression of TTP via regulation of mRNA stability (60). In the presence of a p38 MAPK inhibitor, LPS-induced TTP levels were significantly reduced, but the residual protein was still highly phosphorylated (Fig. 1A and B), suggesting a role for kinases other than p38 MAPK in both the phosphorylation and expression of TTP. To identify kinase pathways involved in regulating TTP expression and phosphorylation, RAW 264.7 cells were treated with LPS in the presence or absence of various kinase inhibitors (Fig. 1A). SP600125 (a Jun N-terminal protein kinase inhibitor), Rottlerin (an inhibitor of several protein kinase C isoforms), or LY294002 (an inhibitor of phosphatidylinositol 3-kinase) did not block the induction of TTP protein by LPS. Only the p38 MAPK inhibitor SB202190 modulated TTP expression. As previously described (60), the MEK1/ERK pathway inhibitor U0126 did not block TTP expression. However, U0126 reproducibly caused an increase in the electrophoretic mobility of TTP, suggesting a reduction in phosphorylation (Fig. 1B; see also Fig. 3 and 4A and B; note that the pattern of migration of TTP bands varies slightly according to conditions of electrophoresis and extract preparation). The inhibition of both the ERK and p38 MAPK pathways by simultaneous addition of U0126 and SB202190 resulted in a complete blockade of LPS-induced TTP expression (Fig. 1B). This synergistic inhibition of TTP expression by simultaneous SB202190 and U0126 addition was not observed when steady-state TTP mRNA levels were examined (Fig. 1C). As previously described, a p38 MAPK inhibitor dose dependently reduced TTP mRNA levels (60), but U0126 had no additive effect at doses that completely block the activation of ERK. These results indicate that the ERK and p38 MAPK pathways synergistically regulate TTP expression at a translational and/or posttranslational level.

TTP protein stability is regulated by the p38 MAPK pathway. To examine the possible posttranslational regulation of TTP levels by p38 MAPK, SB202190 was added to LPS-stimulated RAW 264.7 cells for various times in the presence or absence of cycloheximide (Cx) (Fig. 2A). Between 2 and 4 h after the addition of LPS, the expression of TTP protein increased, but its electrophoretic mobility did not change. In the presence of Cx, the electrophoretic mobility and quantity of TTP remained relatively constant over the same time course, consistent with a reported protein half-life of greater than 4 h (9). In the absence or presence of Cx, addition of SB202190 resulted in a rapid change in electrophoretic mobility of TTP and a decrease in its quantity, consistent with a decrease of protein half-life to approximately 30 min.

Similar experiments were performed using primary murine macrophages (Fig. 2B, left panel) and human monocytes (Fig. 2B, right panel). In each case the addition of SB202190 resulted in complete clearance of TTP protein within two hours in translationally arrested cells. To investigate the role of MK2 in the regulation of TTP protein stability, we performed similar experiments in immortalized macrophages derived from $MK2^{-/-}$ or control mice (Fig. 2C). Note that the expression of TTP was lower in $MK2^{-/-}$ macrophages (26a); therefore, three times as much extract from these cells was loaded for Western blotting. In immortalized wild-type macrophages, TTP protein was relatively stable and again was significantly destabilized following inhibition of the p38 MAPK pathway. TTP was somewhat less stable in immortalized $MK2^{-/-}$ macrophages (compare lanes 2 and 6 as well as 12 and 16). However, significant destabilization was still observed following addition of SB202190. These observations suggest that, in monocytic cell types, LPS-induced TTP is posttranslationally stabilized by the p38 MAPK pathway and that this is mediated, in part, by MK2.

ERK and p38 MAPK pathways synergistically regulate stability and subcellular localization of TTP. We have found that the p38 MAPK pathway regulates subcellular localization of TTP in HeLa cells and primary murine macrophages, promoting its translocation from the nucleus to the cytoplasm (T.

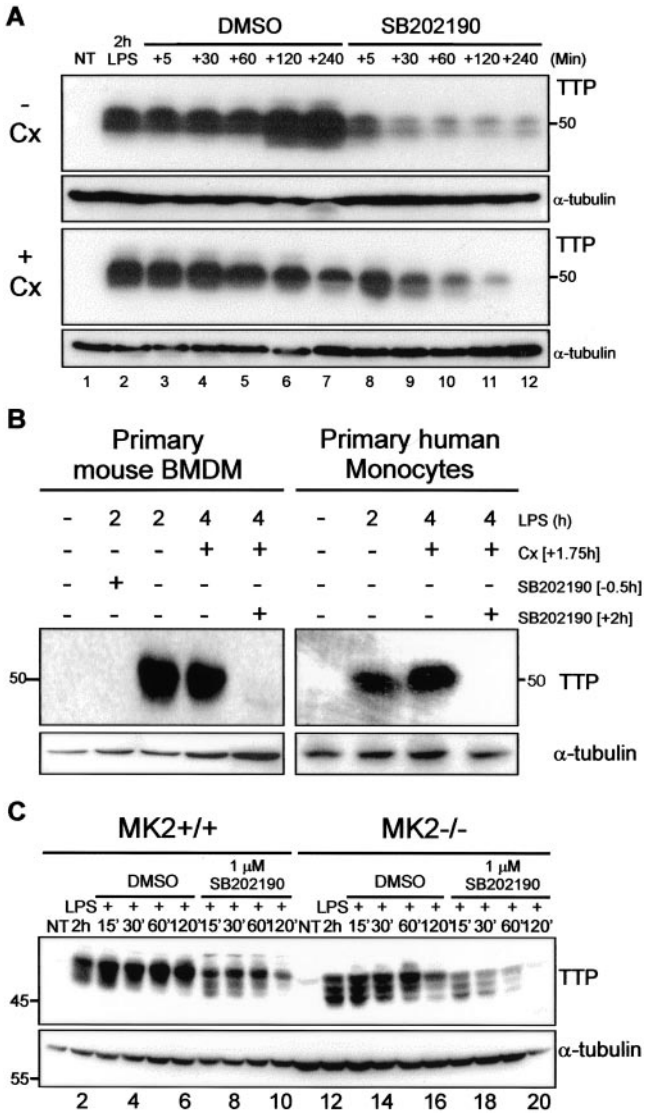

FIG. 2. TTP protein stability is regulated by the p38 MAPK pathway. (A) RAW 264.7 cells were either left unstimulated, stimulated with LPS for 2 h, or stimulated with LPS for 2 h and then treated with 0.1% DMSO or 1 μM SB202190 for the times indicated (upper panel) or stimulated with LPS for 1.75 h and then treated with Cx (5 μg/ml) for 15 min prior to the addition of 0.1% DMSO or 1 μM SB202190 (lower panel) for the times indicated. (B) Primary mouse bone marrow-derived macrophages (BMDM) (left panel) or primary human monocytes (right panel) were either left unstimulated, stimulated with LPS for 2 h in the presence or absence of 1 μM SB202190, or stimulated with LPS for 1.75 h and then treated with Cx (5 μg/ml) for 15 min prior to the addition of 0.1% DMSO or 1 μM SB202190 for a further 2 h. (C) Immortalized wild-type or MK2$^{-/-}$ macrophages were either left unstimulated or stimulated with LPS for 1.75 h and then treated with Cx (5 μg/ml) for 15 min prior to the addition of 0.1% DMSO or 1 μM SB202190 for the times indicated; 100 μg wild type and 300 μg MK2$^{-/-}$ extract were loaded for blotting. In each case (A to C), whole-cell extracts were prepared and TTP and tubulin were detected by Western blotting. Positions of molecular mass markers (in kilodaltons) are indicated. NT, untreated.

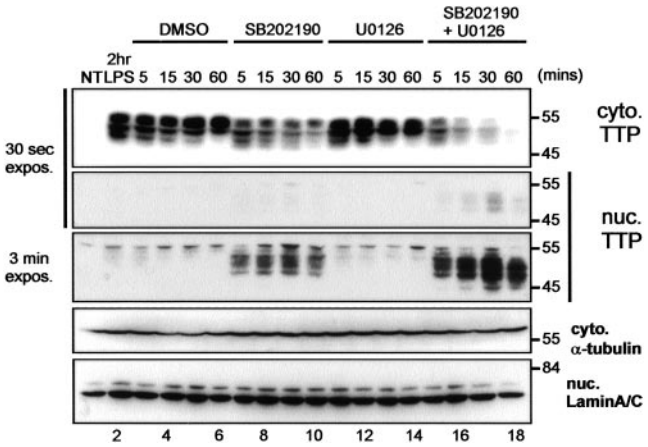

FIG. 3. TTP protein stability and subcellular localization are synergistically regulated by the p38 MAPK and ERK pathways. RAW 264.7 cells were either left unstimulated, stimulated with LPS for 2 h, or stimulated with LPS for 1.75 h and then treated with Cx (5 μg/ml) for 15 min prior to the addition of 0.1% DMSO, 1 μM SB202190, and/or 10 μM U0126 for the times indicated. Cytoplasmic (cyto.) and nuclear (nuc.) extracts were prepared, and TTP, tubulin, and lamin A/C were detected by Western blotting (tubulin was not detected in the nuclear fraction, and lamin A/C was not detected in the cytoplasmic fraction). Positions of molecular mass markers (in kilodaltons) are indicated. Equivalent exposures (expos.) (30 s) of the cytoplasmic and nuclear TTP signals and an extended exposure (3 min) of the nuclear TTP signal are shown. NT, untreated.

Santalucia, M. Brook, E. Hitti, et al., unpublished). As described above, p38 MAPK also regulates the stability of TTP protein in myeloid cells and synergizes with the ERK pathway to regulate expression of TTP at a posttranslational level. We therefore investigated the effects of an inhibitor of the ERK pathway on the stability and subcellular localization of TTP in RAW 264.7 cells.

Following stimulation with LPS for 2 h, vehicle, SB202190, U0126, or both inhibitors were added to cells in the presence of Cx (Fig. 3). Inhibition of p38 MAPK caused a rapid dephosphorylation and an increase in nuclear levels of TTP protein within 5 min, with a reduction of steady-state TTP protein levels at later time points as previously shown. α-Tubulin was blotted as a marker of the cytoplasmic fraction, and lamin A/C was blotted as a marker of the nuclear fraction. Even in very long exposures of Western blots (data not shown), we were unable to detect α-tubulin in the nuclear fraction or lamin A/C in the cytoplasmic fraction, confirming the integrity of the subcellular fractions. Therefore, the p38 MAPK pathway regulates subcellular localization of TTP in RAW 264.7 cells as it does in HeLa cells and primary murine macrophages (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). Inhibition of the ERK pathway resulted in a more subtle change in TTP phosphorylation status and no change in its subcellular distribution or stability. Simultaneous inhibition of both the p38 MAPK and ERK pathways resulted in enhanced nuclear accumulation of TTP relative to that caused by a p38 MAPK inhibitor alone (Fig. 3, compare lanes 7 to 9 with lanes 15 to 17). The electrophoretic mobility of TTP was also increased, suggesting a greater degree of dephosphorylation. Furthermore, the combination of inhibitors caused a synergistic destabilization of TTP protein. In separate experiments employing whole-cell extracts rather than nuclear and cytoplasmic extracts, we estimated that TTP protein was degraded with a half-life between 5 and 10 min in the presence of both

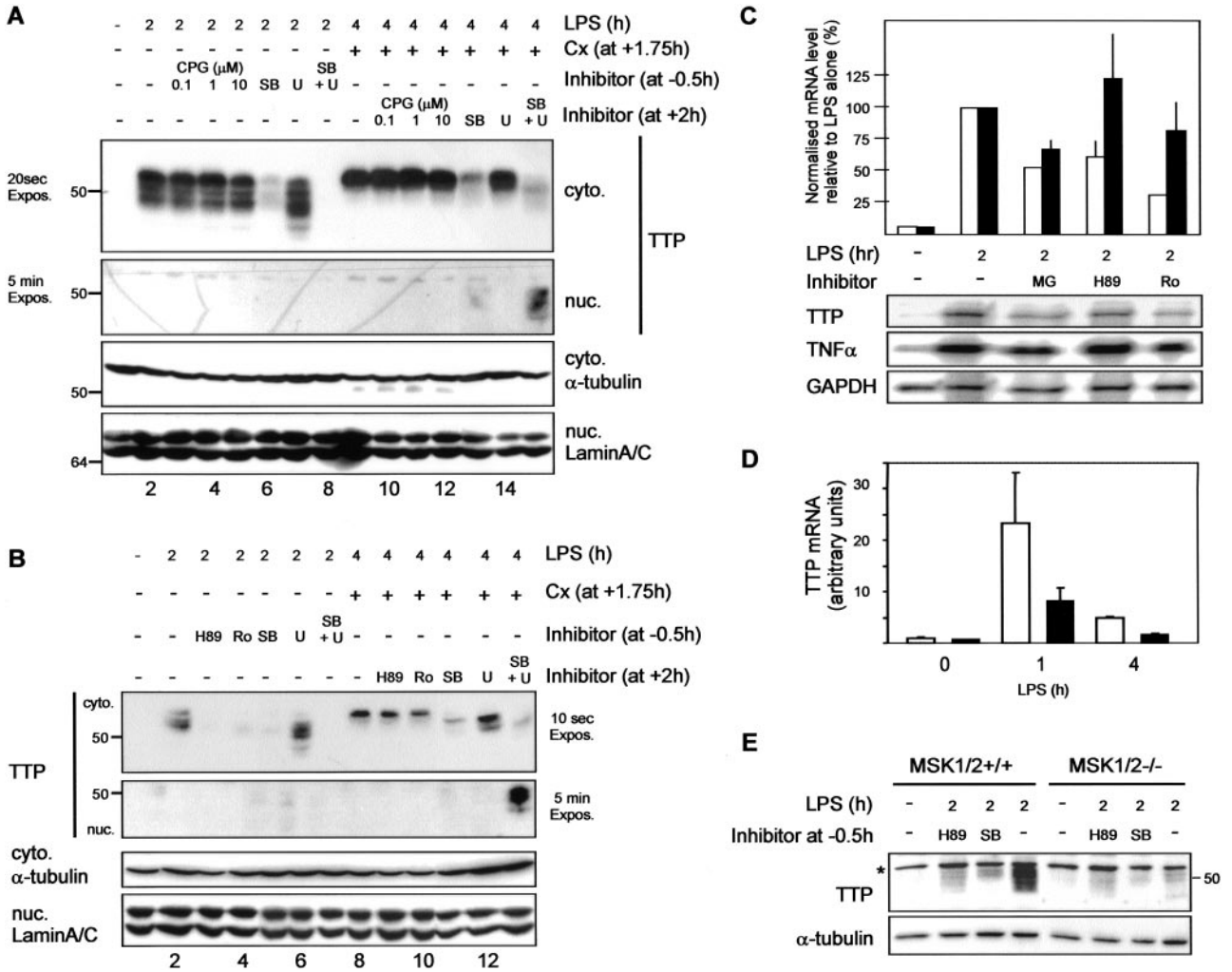

FIG. 4. MSK-1 and -2 but not MNK-1 and -2 regulate TTP protein induction but not its stability or subcellular localization. (A) RAW 264.7 cells were either left unstimulated, stimulated with LPS for 2 h in the presence or absence of the indicated doses of CPG57380 (CPG), 1 μM SB202190 (SB), and/or 10 μM U0126 (U), or stimulated with LPS for 1.75 h and then treated with Cx (5 μg/ml) for 15 min prior to the addition of 0.1% DMSO, the indicated doses of CPG57380, 1 μM SB202190, and/or 10 μM U0126 for the times indicated. An extended exposure (expos.) (5 min) of the nuclear TTP signal is shown. (B) As for panel A, except that the inhibitors used were as follows: 25 μM H89, 5 μM Ro 31-8220, 1 μM SB202190, and/or 10 μM U0126. An extended exposure (5 min) of the nuclear TTP signal is shown. In each case (A and B), cytoplasmic (cyto.) and nuclear (nuc.) extracts were prepared, and TTP, tubulin, and lamin A/C were detected by Western blotting (tubulin was not detected in the nuclear fractions, and lamin A/C was not detected in the cytoplasmic fraction). (C) RAW 264.7 cells were either left unstimulated or treated with 0.1% DMSO, 10 μM MG132 (MG), 25 μM H89, or 5 μM Ro 31-8220 (Ro) for 30 min prior to being treated with LPS for 2 h. Total RNA was prepared, and 4 μg RNA/point was analyzed by RNase protection assay for the expression of TTP mRNA (black bars) and TNF-α mRNA (gray bars). (D) Wild-type (open bars) and MSK-$1^{-/-}$ MSK-$2^{-/-}$ macrophages (black bars) were either left unstimulated or treated with LPS for the times indicated. Total RNA was prepared, and TTP mRNA levels were measured using quantitative reverse transcription-PCR. Means ± standard deviations are shown for duplicate PCRs on two independent macrophage preparations per genotype. (E) Wild-type and MSK-$1^{-/-}$ MSK-$2^{-/-}$ macrophages were either left unstimulated or were treated with 0.1% DMSO, 25 μM H89, or 1 μM SB202190 for 30 min prior to being treated with LPS for 2 h. Whole-cell extracts were prepared for Western blotting. Positions of molecular mass markers (in kilodaltons) are indicated in each case. An asterisk indicates a nonspecific band.

SB202190 and U0126 (data not shown). Thus, the p38 MAPK and ERK pathways synergistically regulate both the subcellular distribution and the stability of TTP protein. Inhibition of these signaling pathways results in rapid dephosphorylation of TTP by an unknown phosphatase or phosphatases, nuclear accumulation, and protein destabilization.

It is known that ERK and p38 MAPK can synergistically activate several downstream kinases, such as MSK-1 and MSK-2 (19, 48) as well as MNK-1 and MNK-2 (30, 63, 64). Therefore, we used inhibitors to investigate the roles of these kinases in the regulation of TTP. CPG57380 inhibits MNK-1 and -2 with a 50% inhibitory concentration of 2.2 μM (30); however, 10 μM CPG57380 did not alter the expression, subcellular localization, or stability of TTP (Fig. 4A), whereas the combination of SB202190 and U0126 caused destabilization and relocalization of TTP protein as before. H89 and Ro 31-8220 inhibit MSK-1 and -2 but do not significantly affect ERK, p38 MAPK, or MK2 (18). Neither of these inhibitors

altered the subcellular localization or stability of TTP (Fig. 4B), suggesting that the synergistic posttranslational regulation of TTP by ERK and p38 MAPK pathways is not mediated by MSK-1 or -2. On the other hand, the induction of TTP protein by LPS was inhibited by either H89 or Ro 31-8220 (Fig. 4B). The induction of TTP mRNA by LPS was also significantly inhibited by either H89 or Ro 31-8220, whereas the induction of TNF-α mRNA was not significantly altered (Fig. 4C). In LPS-stimulated RAW 264.7 cells, the activation of MSK-1 and -2 is principally mediated by p38 MAPK (6). The small input from ERK in this context may account for the lack of inhibition of TTP expression by U0126 alone. MG132, an inhibitor of the proteasome, inhibited the induction of both TNF-α and TTP mRNAs.

H89 and Ro 31-8220 are not highly specific inhibitors of MSK-1 and -2, but they block a number of other signaling pathways (18). More selective inhibitors of other potential H89 and Ro 31-8220 targets did not prevent the induction of TTP mRNA by LPS (data not shown). To provide more conclusive evidence of a role for MSK-1 and -2 in the expression of TTP, we studied LPS-treated bone marrow macrophages derived from an MSK-$1^{-/-}$ MSK-$2^{-/-}$ mouse strain. At both mRNA and protein levels, the expression of TTP was lower in MSK-$1^{-/-}$ MSK-$2^{-/-}$ than in wild-type murine macrophages (Fig. 4D and E). Because of exceptionally weak expression of TTP protein, we were unable to assess the role of MSK-1 and -2 in its posttranslational regulation. Taken together with the inhibitor studies, these data indicate that MSK-1 and/or MSK-2 are required for efficient induction of TTP mRNA but that MSK-1 and -2 or MNK-1 and -2 do not contribute to the synergistic posttranslational regulation of TTP by ERK and p38 MAPK pathways.

TTP protein stability and subcellular localization are regulated by phosphorylation of serines 52 and 178. In vitro, MK2 phosphorylates TTP at serines 52 and 178 (15, 47) (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). Mass spectrometric studies provide evidence for phosphorylation of these sites in vivo (7, 15). This is also supported by differences in electrophoretic mobility between endogenous TTP expressed in wild-type and MK2$^{-/-}$ macrophages (Fig. 2C), GFP-TTP and GFP-TTP-S52A/S178A (Fig. 5B), or Flag-TTP and Flag-TTP-S52A/S178A (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). We have previously described a role for these phosphorylation sites in the regulation of nucleocytoplasmic localization of exogenous TTP in HeLa cells (T. Santalucia, M. Brook, E. Hitti, et al., unpublished). In order to assess the role of these phosphorylations in macrophages, we optimized a protocol for transfection of RAW 264.7 cells. Transfection efficiencies of ~60 to 70% were routinely achieved (as ascertained by flow cytometry and microscopic analysis; data not shown), and as little as 500 ng of plasmid was sufficient to express GFP-tagged TTP at levels similar to those observed for endogenous TTP (data not shown). Unsurprisingly, the electroporation procedure caused transient activation of the p38 MAPK pathway (Fig. 5A), and this was reflected by a weak induction of endogenous TTP expression (Fig. 5B). When RAW 264.7 cells were transfected with a GFP expression vector, fluorescent cells were readily detected after 24 or 48 h. In contrast, when a GFP-TTP expression vector was used, we could scarcely detect fluorescent cells after 16 h (data not shown). To investigate this phenomenon further, we cotransfected RAW 264.7 cells with equal amounts of GFP and GFP-TTP expression vectors and assessed the expression of both proteins over 24 h. When detected using an anti-GFP antibody, GFP and GFP-TTP both comigrated with nonspecific bands (Fig. 5A, upper panel, indicated by an asterisk). However, GFP

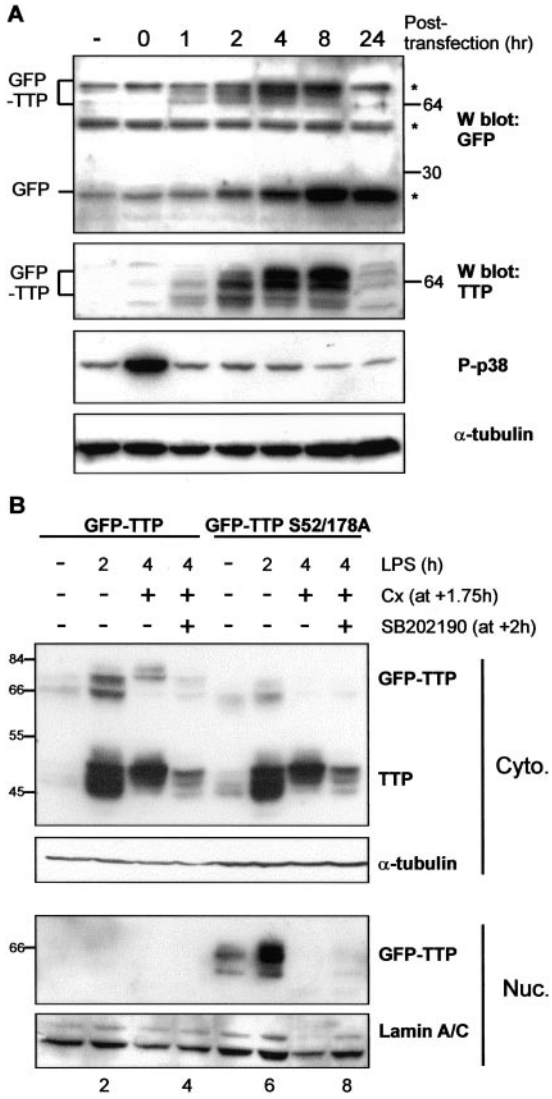

FIG. 5. TTP stability and subcellular localization are regulated by MK2-mediated phosphorylation of serines 52 and 178. (A) RAW 264.7 cells were either left untreated or transfected with peGFPc1-TTP and cultured for the times indicated following transfection. Whole-cell extracts were prepared, and GFP-TTP, GFP, phospho-p38 (P-p38) MAPK, and tubulin were detected by Western blotting (W blot). Asterisks indicate nonspecific bands detected by the anti-GFP antibody. (B) RAW 264.7 cells were transfected with either peGFPc1-TTP or peGFPc1-TTP-S52/178A and then cultured for 2 h. Cells were then either harvested, treated with LPS for 2 h, or treated with LPS for 1.75 h and then treated with 5 μg/ml Cx for 15 min prior to the addition of 0.1% DMSO or 1 μM SB202190 for a further 2 h. Cytoplasmic (cyto.) and nuclear (nuc.) extracts were prepared, and TTP, tubulin, and lamin A/C were detected by Western blotting (tubulin was not detected in the nuclear fractions, and lamin A/C was not detected in the cytoplasmic fraction). Positions of molecular mass markers (in kilodaltons) are indicated.

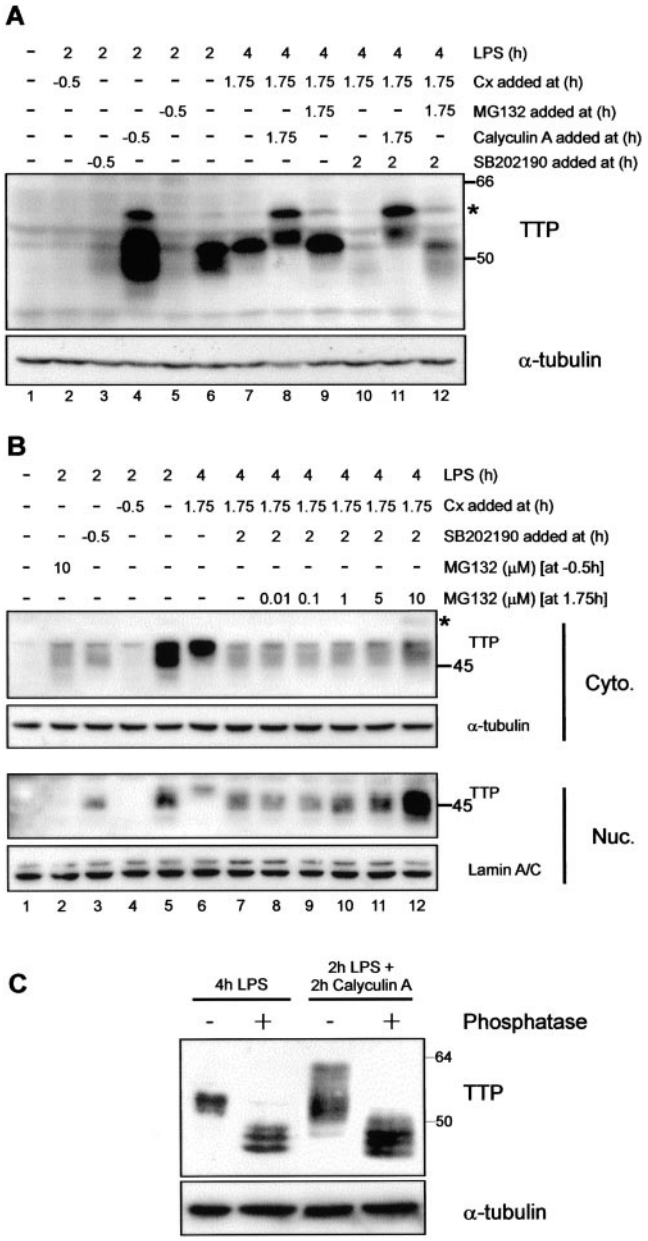

FIG. 6. TTP turnover proceeds via the proteasome and is negatively regulated by p38 MAPK-mediated phosphorylation. (A) RAW 264.7 cells were either left unstimulated, treated with LPS for 2 h in the presence or absence of the indicated inhibitors, or treated with LPS for 1.75 h and then treated with 5 μg/ml Cx and/or 10 μM MG132 or 100 nM calyculin A for 15 min prior to the addition of 0.1% DMSO or 1 μM SB202190 for a further 2 h. Whole-cell extracts were prepared and TTP and tubulin were detected by Western blotting. Positions of molecular mass markers (in kilodaltons) are indicated. (B) RAW 264.7 cells were either left unstimulated, treated with LPS for 2 h in the presence or absence of 5 μg/ml Cx, 1 μM SB202190, or 10 μM MG132, or treated with LPS for 1.75 h and then treated with 5 μg/ml Cx and increasing concentrations of MG132 for 15 min prior to the addition of 1 μM SB202190 for a further 2 h. Cytoplasmic (Cyto.) and nuclear (Nuc.) extracts were prepared, and TTP, tubulin, and lamin A/C were detected by Western blotting (tubulin was not detected in the nuclear fraction, and lamin A/C was not detected in the cytoplasmic fraction). (C) RAW 264.7 cells were either LPS stimulated for 4 h or treated with LPS for 2 h and then treated with 10 nM calyculin A for a further 2 h. Complete cell extracts were prepared and either left untreated or incubated with 50 U shrimp alkaline phosphatase for 2 h at 37°C prior to Western blotting. Positions of molecular mass markers (in kilodaltons) are indicated. An asterisk indicates a novel ~63-kDa form of TTP.

was detectable from 2 h posttransfection and was sustained for at least 24 h, whereas expression of GFP-TTP was weak and declined after 8 h. The expression pattern of GFP-TTP was confirmed using an anti-TTP antibody (Fig. 5A). This observation suggests that the decline in expression of GFP-TTP is not caused by apoptosis of transfected cells but may be related to the stability of the GFP-TTP fusion protein.

GFP-TTP was expressed at low levels in unstimulated RAW 264.7 cells (Fig. 5B) and was significantly upregulated and phosphorylated following treatment with LPS for 2 h. GFP-tagged TTP in which serines 52 and 178 were mutated to alanine (GFP-TTP-S52/178A) was consistently expressed at lower levels than GFP-TTP and was only weakly upregulated and phosphorylated upon LPS stimulation (Fig. 5B). The selective upregulation of GFP-TTP but not GFP-TTP-S52A/S178A rules out a transcription-mediated response and suggests different posttranslational processing of the two proteins. Like endogenous TTP protein, GFP-TTP remained relatively stable for 2 h following the addition of Cx, but it was rapidly degraded if the p38 MAPK pathway was simultaneously inhibited. In contrast, GFP-TTP-S52A/S178A was degraded rapidly whether the p38 MAPK pathway was inhibited or not. The two proteins also behaved differently in terms of their subcellular localization: GFP-TTP was almost undetectable in the nucleus, whereas GFP-TTP-S52A/S178A was readily detected in the nuclear fraction. Hence, the posttranslational regulation of TTP protein stability and subcellular localization is, at least partly, dependent on the integrity of serine 52 and/or 178.

TTP degradation requires phosphatase activity and proceeds via the proteasome. LPS-induced TTP protein is rapidly dephosphorylated following p38 MAPK inhibition, suggesting that TTP is subject to dynamic regulation by kinase and phosphatase activities. The dephosphorylated TTP is rapidly degraded, leading us to hypothesize that the protein may be targeted for destruction by the 20S/26S proteasomes. The mechanism of TTP turnover was investigated further using inhibitors specific for serine/threonine (ser/thr) phosphatases (calyculin A) or the 20S/26S proteasomes (MG132). RAW 264.7 cells were either left unstimulated, stimulated with LPS for 2 h in the presence or absence of inhibitors, or stimulated with LPS for 2 h prior to addition of vehicle or inhibitor in the presence of Cx (Fig. 6A). The LPS-induced expression of TTP protein was enhanced by calyculin A (Fig. 6A, lanes 1, 4, and 6), possibly due to increased activation of the p38 MAPK signaling pathway (data not shown). In contrast, TTP induction was blocked by Cx, SB202190, or MG132 (Fig. 6A, lanes 2, 3, and 5). TTP protein was relatively stable for the duration of the 2-h translational arrest but was destabilized by addition of SB202190 (Fig. 6A, compare lanes 6, 7, and 10). This destabilization was significantly reduced in the presence of either phosphatase or proteasome inhibitor (Fig. 6A, compare lane 10 to lane 12). Thus, we conclude that the p38 MAPK pathway phosphorylates and stabilizes TTP and that acute inhibition of p38 MAPK causes rapid TTP dephosphorylation by an un-

known ser/thr phosphatase. Once dephosphorylated, the TTP is targeted to the proteasome by an unknown mechanism and rapidly degraded.

Interestingly, in the presence of both SB202190 and MG132, the majority of TTP protein exhibited increased electrophoretic mobility (Fig. 6A, compare lane 7 to lane 12), suggesting that the proteasome inhibitor may protect a dephosphorylated form of TTP from degradation. We hypothesized that the dephosphorylated TTP would have an altered subcellular distribution. To test this hypothesis, we prepared nuclear and cytoplasmic extracts from RAW 264.7 cells that were either untreated, stimulated with LPS for 2 h in the presence or absence of inhibitors, or stimulated with LPS for 2 h before addition of vehicle or inhibitors in the presence of Cx for a further 2 h. After 2 h of LPS treatment, TTP was largely cytoplasmic and phosphorylated (Fig. 6B, lanes 1 and 5). After a further 2 h of translational arrest, TTP levels were unchanged and the protein was highly phosphorylated and predominantly cytoplasmic (Fig. 6B, lanes 5 and 6). The combination of Cx and SB202190 resulted in dephosphorylation and degradation of TTP (Fig. 6B, lanes 6 and 7). The addition of MG132 to these cells caused a dose-dependent reversal of the TTP-destabilizing effects of SB202190, with 10 μM MG132 blocking the effects of p38 MAPK inhibition. However, this protected TTP was dephosphorylated and predominately nuclear (Fig. 6B, compare lanes 6 and 12).

In extracts of calyculin A-treated cells, and more weakly in MG132-treated cells, we detected a low-mobility TTP form of an apparent molecular mass of ~63 kDa (identified by an asterisk in Fig. 6A). Phosphatase treatment of cell extracts caused collapse of all TTP bands, including the 63-kDa band, to faster migrating positions, suggesting that the 63-kDa form of TTP is hyperphosphorylated. However, the TTP bands did not collapse to a single position. We conclude that either some phosphorylations are resistant to dephosphorylation in vitro or additional posttranslational modifications of TTP remain to be identified.

DISCUSSION

The p38 MAPK signaling pathway regulates expression of many inflammatory mediators at a posttranscriptional level via MK2-mediated stabilization of otherwise highly labile mRNAs (16, 22). At least some of these inflammatory mediators are also known to be posttranscriptionally controlled by TTP, a well-characterized mRNA destabilizing factor. The reasons for studying functional links between p38 MAPK and TTP are therefore clear, yet there remains controversy over whether TTP is a substrate of p38 MAPK itself, MK2, or both (8, 10, 15, 47, 67); whether or not such phosphorylation alters TTP function (52, 56) (T. Santalucia, M. Brook, E. Hitti, et al., unpublished); and whether or not it results in recruitment of 14-3-3 proteins (15, 52, 56). Several previous studies have been performed using cell types that express little or no endogenous TTP and in which overexpression of the protein may subvert the normal regulation of stability, localization, or function. With this in mind, here we performed experiments using RAW 264.7 cells, primary monocytes, or macrophages that all express TTP in response to LPS. We also optimized a transfection protocol that allowed expression of exogenous TTP at levels similar to those of endogenous protein.

We describe evidence that the p38 MAPK signaling pathway regulates the stability of TTP protein in myeloid cells via MK2-mediated phosphorylation of serines 52 and 178. (i) In cycloheximide chase experiments performed in RAW 264.7 cells, a p38 MAPK inhibitor destabilized endogenous TTP protein, reducing its half-life from >4 h to approximately 30 min. (ii) In primary human monocytes or murine macrophages, endogenous TTP protein was even more strikingly destabilized following inhibition of p38 MAPK. (iii) LPS-induced TTP protein displayed weaker expression and lower stability in immortalized $MK2^{-/-}$ macrophages than in wild-type control cells. (iv) In transfected RAW 264.7 cells, a GFP-TTP fusion protein was expressed only transiently. This was correlated with a transient activation of the p38 MAPK pathway caused by the transfection procedure. (v) Expression of GFP-TTP was further enhanced by stimulation of RAW 264.7 cells with LPS. This upregulation was not observed with a GFP-TTP fusion protein lacking the major MK2 phosphorylation sites, and it was inhibited by SB202190 (data not shown). We observed little or no LPS-induced increase in expression of GFP-TTP in transfected $MK2^{-/-}$ macrophages, but unfortunately we could not transfect the paired wild-type macrophages to provide an appropriate control for this experiment (data not shown). (vi) In transfected RAW 264.7 cells, GFP-TTP protein was strongly destabilized by SB202190, whereas the mutant protein lacking the MK2 phosphorylation sites was inherently unstable in the absence or presence of SB202190.

There is also evidence that TTP stability may be regulated by p38 MAPK in an MK2-independent manner, since SB202190 still destabilizes TTP protein in $MK2^{-/-}$ cells. Furthermore, we have shown that TTP stability is synergistically regulated by ERK and p38 MAPK pathways. This posttranscriptional synergy was not mediated by downstream kinases MSK-1 or -2 as well as MNK-1 or -2, which are known to be activated by both ERK and p38 MAPK. A recent publication describes the identification of several sites of phosphorylation of TTP in vivo, many of which are potential targets for members of the MAPK pathway (7). Our future experiments will determine whether the stability of TTP is modulated via direct phosphorylation by ERK or p38 MAPK. Serine 220 was previously identified as a putative site of direct phosphorylation of TTP by ERK, but no function has yet been ascribed to this phosphorylation (36, 58, 59).

In RAW 264.7 cells that had been treated with LPS for 2 h, the addition of SB202190 caused a rapid change in the electrophoretic mobility of endogenous TTP that was indicative of dephosphorylation and preceded TTP degradation. Calyculin A, an inhibitor of serine/threonine phosphatases, protected TTP from SB202190-induced degradation and caused accumulation of a low-mobility, apparently hyperphosphorylated form of the protein. Hence, the phosphorylation status of TTP depends on a dynamic equilibrium of kinase and phosphatase activities, dephosphorylation is a prerequisite for protein degradation, and the behavior of TTP is exquisitely sensitive to the status of the p38 MAPK pathway in myeloid cells that have been exposed to LPS. We are attempting to identify the putative TTP phosphatase, which presumably plays an important

role in determining the course of the inflammatory response. Importantly, in vitro phosphatase treatment of cell extracts did not resolve TTP to a single, discrete band, therefore additional posttranslational modifications of TTP may also remain to be identified.

MG132 prevented the degradation but not the dephosphorylation of TTP following inhibition of the p38 MAPK pathway; therefore, a hypophosphorylated form of TTP is targeted for degradation by the 20S/26S proteasome. This contrasts with other examples such as IκB, in which phosphorylation precedes polyubiquitination and degradation, leading to activation of the transcription factor NF-κB. Rigby et al. (52) recently described the existence of putative protein stability-regulating PEST domains in human TTP. While point mutation of these domains did not affect the steady-state expression levels of TTP in HEK 293 cells, the phosphorylation profile of the proteins was altered. It will be of interest to examine the role of the PEST domains and their phosphorylation in the p38 MAPK-mediated regulation of TTP stability in monocytic cell types. MG132 was previously shown to inhibit the decay of ARE-containing mRNAs, suggesting some form of coupling between turnover of proteins and mRNAs (42, 43). We were struck by the similarity in rates of clearance of TTP protein and TNF-α mRNA in SB202190-treated RAW 264.7 cells and by the observation that ERK and p38 MAPK pathways synergistically regulate the stability of both TTP protein (this work) and TNF-α mRNA (53). However, our preliminary experiments revealed that MG132 decreased rather than increased the stability of TNF-α mRNA (data not shown). Therefore, the turnover of TTP protein and TNF-α mRNA does not appear to be directly coupled in myeloid cells. The mechanisms described here are more likely to play a role in the tight regulation of cellular TTP levels.

In a parallel study (T. Santalucia, M. Brook, E. Hitti et al., unpublished), we showed that p38 MAPK-dependent phosphorylation of serines 52 and 178 regulated the relocalization of TTP from the nucleus to the cytoplasm in transfected HeLa cells. It proved difficult to study this phenomenon in myeloid cells because of the SB202190-induced destabilization of TTP protein. However, our results are consistent with a similar regulation of subcellular localization in these cells. (i) In LPS-treated RAW 264.7 cells, the addition of SB202190 (alone or in combination with U0126) caused a rapid increase in nuclear TTP, accompanied by a change in electrophoretic mobility that indicated dephosphorylation. (ii) In transfected RAW 264.7 cells, GFP-TTP was almost exclusively cytoplasmic, but a mutant form of this protein lacking the MK2 phosphorylation sites was detected in both cytoplasmic and nuclear compartments. In transfected immortalized MK2$^{-/-}$ macrophages, GFP-TTP and the S52/178A mutant were expressed equally strongly in the nucleus, suggesting that MK2 activity is required for differential localization (data not shown). (iii) Most compellingly, the dephosphorylation of TTP could be uncoupled from its degradation using a proteasome inhibitor. Under these conditions, TTP was hypophosphorylated and almost exclusively nuclear. Although the regulation of TTP subcellular localization by ERK and p38 MAPK is clear, the function and mechanism of this regulation are uncertain. It is possible that TTP destabilizes many unidentified mRNAs containing the consensus binding site UUAUUUAUU (38, 65), several of which encode

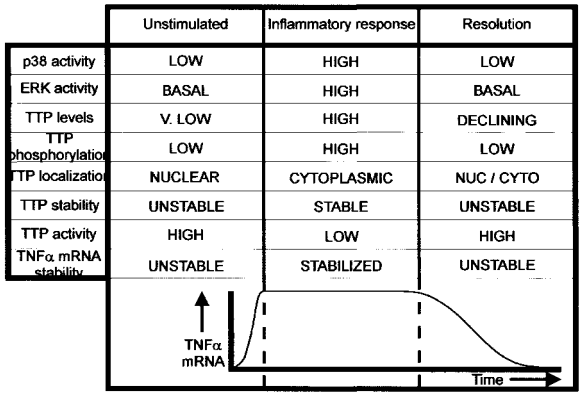

FIG. 7. Model for the regulation of inflammation by p38 MAPK and TTP. NUC, nuclear; CYTO, cytoplasmic; V, very.

regulators of growth and apoptosis (1). We speculate that the cytoplasmic localization of TTP is tightly regulated in order to protect cells from the well-characterized proapoptotic consequences of prolonged expression (27, 28). The cytoplasmic localization of S52/178-phosphorylated TTP may be mediated by the binding of 14-3-3 proteins (15, 56). Since 14-3-3 proteins are known to regulate both the localization and stability of interacting proteins (4, 61, 66), it will be of interest to examine the role of this interaction in the regulation of TTP turnover.

In the course of these studies, we made some observations pertaining to the transcriptional control of TTP expression. First, efficient expression of TTP mRNA was dependent on MSK-1 and/or MSK-2 being blocked by inhibitors of these kinases and impaired in murine MSK-1$^{-/-}$ MSK-2$^{-/-}$ bone marrow macrophages. TTP may be added to a list of immediate-early genes, including c-*fos*, *junB*, and the nuclear orphan receptor *Nur77*, whose expression requires the MSKs (17). Second, a proteasome inhibitor prevented the induction of TTP protein and impaired the induction of TTP mRNA in response to LPS. A possible interpretation is that TTP transcription is regulated by NF-κB, since the TTP intron is required for correct regulation of gene expression and contains a conserved NF-κB consensus sequence (41).

Paradoxically, the p38 MAPK pathway stabilizes proinflammatory mRNAs but also stabilizes TTP protein, which has the opposite effect. In Fig. 7, we present a model for the regulation of TTP biology by the p38 MAPK pathway during the distinct phases of an inflammatory response. In unstimulated cells, containing little or no p38 MAPK activity, TTP is expressed at low (undetectable) levels and is likely to be hypophosphorylated, unstable, and principally nuclear in localization. Upon stimulation with an inflammatory agonist, p38 MAPK is activated and expression of both TNF-α and TTP is upregulated. Expression of TTP is tightly coupled to activation of the p38 MAPK pathway via the regulation of mRNA stability and protein stability, so that the protein accumulates under conditions that favor its phosphorylation. However, TTP phosphorylation impairs its destabilizing function, thereby ensuring that TNF-α mRNA can be expressed and translated. While the p38 MAPK pathway remains active, TTP remains stable, cytoplasmic, and inactive. During the resolution of inflammation, p38 MAPK activity declines and TTP is dephosphorylated and

activated, causing the destabilization of TNF-α mRNA. At the same time, TTP protein begins to relocalize to the nucleus and/or be degraded by the 20S/26S proteasome. This may protect the cell from proapoptotic (27) or other undesired effects of prolonged TTP expression and/or reestablish conditions in which a response to a second proinflammatory stimulus can be mounted. In this model, the p38 MAPK pathway contributes to both on and off phases of the inflammatory response. Such a fail-safe mechanism would help to ensure that disregulation of the p38 MAPK pathway alone is not sufficient for inappropriate proinflammatory gene expression.

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