Immunological Characterization of Tristetraprolin as a Low Abundance, Inducible, Stable Cytosolic Protein*  

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Tristetraprolin (TTP) is a zinc finger protein that can bind to AU-rich elements within certain mRNAs, resulting in deadenylation and destabilization of those mRNAs. Its physiological targets include the mRNAs encoding the cytokines tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor. TTP was originally identified on the basis of its massive but transient increase in mRNA levels following mitogen stimulation of fibroblasts. It has been difficult to reconcile this transient mRNA profile with the presumed continuing “need” for TTP protein, for example, to reverse the effects of lipopolysaccharide (LPS)-stimulated TNF secretion. To investigate this and other questions concerning endogenous TTP protein in cells and tissues, we raised a high titer rabbit antiserum against full-length mouse TTP. TTP could be detected on immunoblots of mouse cytosolic tissue extracts; it was most highly expressed in spleen, but its concentration in that tissue was only about 1.5 nM. TTP could be detected readily in spleen and serum of mice from LPS-injected rats. In both LPS-treated RAW 264.7 macrophages and fetal calf serum-treated mouse embryonic fibroblasts, TTP protein was stable after induction, with minimal degradation occurring for several hours after treatment of the cells with cycloheximide. The biosynthesis of TTP was accompanied by large changes in electrophoretic mobility consistent with progressive phosphorylation. Confocal microscopy revealed that TTP accumulated in a vesicular pattern in the cytosol of the LPS-stimulated RAW 264.7 cells, and was occasionally seen in the cytosol of unstimulated dividing cells. Gel filtration of the endogenous protein suggested that its predominant structure was monomeric. TTP appears to be a low abundance, cytosolic protein in unstimulated cells and tissues, but once induced is relatively stable, in contrast to its very labile mRNA.

Tristetraprolin (TTP)* is the best-understood member of a small family of tandem CCCH zinc finger proteins. In mammals, three members of this family have been characterized: TTP (also known as ZFP36, TIS11, G0S24, and NUP475), ZFP36L1 (also known as TIS11b, cMG1, ERF1, BRF1, and Berg36), and ZFP36L2 (also known as TIS11d, ERF2, and BRF2) (1). Although they are encoded by different genes and their patterns of cell- and tissue-specific expression and agonist-stimulated expression are quite different, they share certain properties: All have highly conserved tandem zinc finger domains, in which each C8x5xC3xH zinc finger is preceded by an AU-rich element (ARE) within single-stranded RNA (2, 3–4) and promoting the deadenylation and subsequent destruction of those transcripts, both in transfection studies and in cell-free experiments (2, 7, 8, 10). In intact animals, TTP deficiency causes a profound inflammatory syndrome with erosive arthritis, autoimmunity, and myeloid hyperplasia, apparently due almost entirely to excessive production of tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), both of whose mRNAs are direct targets of TTP and are stabilized in cells from the knockout (KO) mice (7, 10, 11). For these reasons, TTP can be thought of as an anti-inflammatory or arthritis-suppressing protein.

The cDNAs encoding TTP were originally cloned by three groups by virtue of its very rapid and dramatic transcriptional induction in fibroblasts in response to insulin, phorbol esters, and serum (12–14). In a typical example, TTP mRNA was undetectable in serum-starved, insulin-responsive 3T3-L1 fibroblasts, but the transcript became detectable within 10 min of insulin stimulation, peaked at ~45 min, and returned to near baseline by 120 min (13). These dramatic but transient responses were also seen in cells in which TTP is thought to exert an effect in normal physiology, i.e. in the regulation of TNF mRNA stability in macrophages (9). Clearly, in most cell types there is a mechanism for the rapid transcription of the TTP gene (Zfp36 in the mouse) in response to various agonists, mediated by some well characterized and some relatively uncharacterized enhancer elements (15), and a mechanism for the rapid turning off of transcription while transcript degradation is occurring, leaving quickly to a return to baseline mRNA levels despite the continued presence of agonist (1).

This rapid but transient increase in TTP mRNA levels is difficult to reconcile with the presumed continuing requirement for TTP protein in situations in which it might be needed to destabilize TNF mRNA, for example. For these and other reasons, TTP can be thought of as an anti-inflammatory or arthritis-suppressing protein.

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‡ The abbreviations used are: TTP, tristetraprolin; hTTP, human TTP; mTTP, mouse TTP; ARE, AU-rich element; BMM, mouse bone marrow-derived macrophages; BSA, bovine serum albumin; CHX, cycloheximide; CIAP, calf intestine alkaline phosphatase; FCS, fetal calf serum; GAR-HRP, goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate; GM-CSF, granulocyte-macrophage colony-stimulating factor; KO, knockout; LPS, lipopolysaccharide; MBP, maltose binding protein; MEF, mouse embryonic fibroblasts; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TNF, tumor necrosis factor alpha; TNFR, TNF receptor; TTBS, Tween (0.05%) in Tris-buffered saline; WT, wild-type.

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reasons, it was important to establish the normal patterns of TTP protein expression in cells and tissues, as well as the protein response patterns in cells such as macrophages in which the protein is thought to play an important physiological role. However, studies of this type have been hampered by the lack of good antibodies to TTP, a deficiency that is partly due to difficulties in expressing the intact protein in soluble form in typical expression systems (6). Here, we describe the development of a high titer antibody to mouse TTP, and its use in characterizing the normal patterns of TTP expression in mouse tissues and its pattern of induction in cultured cells. Among the interesting findings were that TTP seems to be an extraordinarily low abundance protein in normal mouse tissues; that its levels do not necessarily parallel the steady-state levels of its transcript in these tissues; that it is a very stable cytoplasmic protein once induced, at least in macrophages and fibroblasts; and finally, that it undergoes progressive phosphorylation during biosynthetic induction over a scale of hours.

MATERIALS AND METHODS

MBP-mTTP Purification and Antibody Production—Maltose binding protein-mouse TTP fusion protein (MBP-mTTP) and MBP were purified as described (6) and used to produce rabbit antisera according to standard procedures (Covance Research Products, Inc., Denver, PA). The antiserum that contained the highest titer anti-TTP activity was centrifuged at 10,000 × g for 10 min, sodium azide was added to a final concentration of 0.02% (w/v), and the serum was stored at −70 °C.

Preparation of Tissue Extracts—Wild-type (WT) C57B16 mice, and mice deficient in TTP, TTP/TNFα−/− (7) were maintained in a barrier facility according to institutional guidelines. Tissues were collected from mice at ~6 months of age after euthanasia with CO2. The tissues were frozen in liquid nitrogen, stored at −70 °C, pulverized with a mortar and pestle under liquid nitrogen, and then homogenized with an Overhead Strirrer (Wheaton Instruments, Millville, NJ) in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1.5 mM MgCl2, 250 mM sucrose, 100 mM NaF, 5 mM diithiothreitol, 2 mM sodium phosphate buffer, 2 mM ZnCl2, 1 mM phenylmethylsulfonyl fluoride, and 2 μM leupeptin. Supernatants from centrifugations at 20,000 × g for 10 min at 4 °C were stored at −70 °C for future use. The protein standards (Amersham Biosciences) used were bovine pancreas ribonuclease A (13.7 kDa), bovine pancreas chymotrypsinogen (25 kDa), hen egg ovalbumin (43 kDa), bovine serum albumin (67 kDa), rabbit muscle aldolase (158 kDa), bovine liver catalase (232 kDa), horse spleen ferritin (440 kDa), and bovine thyroid thyroglobulin (689 kDa). The void volume of the column was determined with blue dextran (2000 kDa).

SDS-PAGE and Immunoblotting—Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (BSA) as the standard, with NaOH treated proteins as controls (21). Proteins in the 10,000 × g supernatant from RAW 264.7 cells were separated on a Superose 6 HR 10/30 size exclusion column (Amersham Biosciences, Uppsala, Sweden) using a similar procedure to that described previously (22). Proteins were eluted with 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8.0, and column fractions were analyzed by immunoblotting using anti-MBP-mTTP serum. The molecular mass of proteins was determined by comparing their elution volume to a standard curve generated with proteins standards separated on the same column. The protein standards (Amersham Biosciences) used were bovine pancreas ribonuclease A (13.7 kDa), bovine pancreas chymotrypsinogen (25 kDa), hen egg ovalbumin (43 kDa), bovine serum albumin (67 kDa), rabbit muscle aldolase (158 kDa), bovine liver catalase (232 kDa), horse spleen ferritin (440 kDa), and bovine thyroid thyroglobulin (689 kDa). The void volume of the column was determined with blue dextran (2000 kDa).

Immunocytochemistry and Confocal Microscopy of RAW 264.7 Cells—Mouse RAW 264.7 cells were grown on glass slides or glass coverslips under the conditions described above. Following the addition of LPS (0.1 μg/ml) for various times, the cells were washed with ice-cold PBS three times before being fixed at room temperature for 10 min in 4% (w/v) paraformaldehyde in PBS. Cells were again washed with PBS twice with 0.2% (w/v) Triton X-100 and fixed for an additional 30 min at room temperature for 10 min. The cells were then incubated at 4 °C overnight in anti-MBP-mTTP serum (1:8 dilution) and 1% normal goat serum in phosphate buffered saline (TTBS) for 30–60 min and incubated with the primary antibody at a 1:10,000 dilution in the blocking buffer for 1–18 h. After being washed with TTBS three times for 10 min each, the membranes were incubated with a 1:10,000 dilution of secondary antibody in TTBS for 1–6 h. Following three, 10-min washes with TTBS, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5–10 min and exposed to x-ray film. The sections were then washed with anti-MBP serum (New England Biolabs, Beverly, MA) and the anti-MBP-mTTP antibody described here and its pre-immune control serum. The secondary antibodies were affinity-purified goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate with human IgG adsorbed (GAR-HRP) (Bio-Rad).

Immunostaining of Rat Spleen Sections—We took advantage of the fact that mice and rat TTP are ~96% identical (compare GenBank accession numbers NP_035886 and NP_579824) to examine the patterns of immunoreactive TTP expression in rat spleen. Male Sprague-Dawley rats at ~12 weeks of age were maintained as described (23) and injected intraperitoneally with LPS (5 mg/kg in 5 ml/kg PBS) or the same volume of PBS as a control. 2 h later, spleens were collected, fixed in 4% (w/v) paraformaldehyde for 48 h, then embedded in paraffin and used for immunohistochemistry essentially as described (23). The sections were incubated with the anti-MBP-mTTP serum or preimmune serum (1:1000) in PBS containing 1% BSA at 37 °C for 45 min, washed in PBS three times for 5 min each, and incubated with a biotin-labeled anti-rabbit antibody (1:200) (Vector Laboratories, Burlingame, CA) in PBS containing 1% BSA at 37 °C for 20 min. The sections were washed as above, and the avidin/biotin complex was developed with 3,3′-diaminobenzidine staining, counterstaining, and dehydration as described (25) using the Vectastain ABC Elite kit (Vector Laboratories).
Identification of TTP in Mouse Cells and Tissues—Using the present antiserum at 1:10,000 dilution, TTP could be detected in Western blots of normal spleen when 5 mg of protein per gel lane and the SuperSignal detection reagent were used (Fig. 2A). TTP in cells and tissues occurs as multiple bands of $M_r$ about 40–50,000, probably a mixture of differentially phosphorylated species and degradation products (1, 7). The TTP bands were absent in spleen taken from TTP KO mice (Fig. 2B). As a positive control, we used 2 µg of protein extract from transfected 293 cells (Fig. 2A).

The 20,000 × g spleen supernatant was separated into supernatant and pellet fractions by centrifugation at 100,000 × g. TTP was found primarily in the 100,000 × g supernatant, with very little immunoreactivity in the membrane pellet (Fig. 2B). No TTP signal was detected in the 20,000 × g pellet (data not shown). Because purified recombinant MBP-mTTP (10 ng, or 0.134 pmol) of the 74.9-kDa fusion protein was found on the same blot as the spleen cytosol (1.7 mg of total protein), we used this blot to estimate the approximate concentration of TTP in normal mouse spleen. In these studies, the average spleen weight was 95.2 mg ($n = 14$), the average soluble protein content per spleen was 16.5 mg/spleen ($n = 14$), and adult mouse spleen water content was ~86% (26). As shown in Fig. 2B, the TTP immunoreactivity in 1.7 mg of spleen extract protein appeared to be about 10% of that of 10 ng (0.134 pmol) of the recombinant protein, or 0.0134 pmol. Therefore, TTP was expressed at −0.0134 pmol per 1.7 mg/16.5 mg/spleen, or 0.1265 pmol/spleen. Because average spleen water content was 86% of an average wet weight of 95.2 mg/spleen, the final estimated TTP concentration was 0.1265 pmol/81.87 μl, or about 1.5 nM.

RESULTS

Production and Characterization of Anti-MBP-mTTP Serum—For antigen production, MBP-mTTP was expressed in Escherichia coli (6) and purified to near-homogeneity as described using the SuperSignal detection reagent and a 5-s exposure of the blot (Fig. 1C). The positions of MBP-mTTP and MBP are indicated. B, detection of MBP-mTTP and MBP by anti-MBP serum. The samples were identical to those shown in A except that about 10% of the amount of protein was used in each lane. C, characterization of anti-MBP-mTTP serum by Western blotting. The indicated amounts of MBP-mTTP eluted from the amylose resin column, and HA-mTTP in soluble extracts of transfected 293 cells, were probed with the anti-MBP-mTTP serum (1:10,000) and GAR-HRP (1:10,000) for 30 min each before being incubated in the detection reagent for 5 min and exposed to x-ray film for 30 s. The smaller immunoreactive bands seen both with the E. coli protein and the protein expressed in 293 cells are presumed to be proteolytic fragments.

Identification of TTP in Mouse Cells and Tissues—Using the present antiserum at 1:10,000 dilution, TTP could be detected in Western blots of normal spleen when 5 mg of protein per gel lane and the SuperSignal detection reagent were used (Fig. 2A). TTP in cells and tissues occurs as multiple bands of $M_r$ about 40–50,000, probably a mixture of differentially phosphorylated species and degradation products (1, 7). The TTP bands were absent in spleen taken from TTP KO mice (Fig. 2B). As a positive control, we used 2 µg of protein extract from transfected 293 cells (Fig. 2A).

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Distribution of TTP in Mouse Tissues—The anti-MBP-mTTP serum (1:10,000 dilution) cross-reacted with proteins of the appropriate size in spleen, lung, liver, large intestine, and thymus, and with proteins of different sizes from several other tissues (Fig. 3A). To distinguish between true positive and false positive signals, we analyzed tissues from mice deficient in either TNFR1 and WT for TTP (Fig. 3B) or deficient in both TNFR1 and TTP (Fig. 3C). These genotypes were used, because the absence of the TNFR1 largely prevents the development of the inflammatory TTP-deficiency syndrome, which might have altered the expression of non-specific bands in the tissues. When compared with tissues from the TTP KO mice (Fig. 3C), true positive TTP signals were seen in spleen, lung, liver, and large intestine (Fig. 3B), whereas non-specific immunoreactive bands of various sizes were detected in brain, heart, pancreas, and skin from both the TTP WT and KO mice (Fig. 3, compare B and C). These data showed that spleen expressed the highest concentration of TTP of any tissue examined.

Induction of TTP in Cultured Cells—To address the specificity of this antiserum in cultured cells, BMM from WT and TTP KO mice were exposed to LPS (1 µg/ml) for 2 h, and proteins from 10,000 × g supernatants were separated by SDS-PAGE and probed with the antiserum at a 1:10,000 dilution. The membrane was incubated in anti-MBP-mTTP serum (1:10,000) overnight and with GAR-HRP (1:10,000) for 1 h and exposed to x-ray film for 1 min. B, the 20,000 × g supernatant of mouse spleen was further centrifuged at 100,000 × g for 1 h to separate microsomal membranes (Membranes) from the cytosol (Soluble). Equal amounts of protein (1.7 mg) from the soluble and membrane fractions were used for Western blotting as above, along with MBP-mTTP (10 ng) from the amylose resin column and HA-mTTP in the transfected 293 cell extracts (1 µg of total soluble protein). In this case, the membrane was exposed to x-ray film for 10 min.

Time Course of Induction and Stability of TTP in Cultured Cells—To investigate the patterns of TTP protein accumulation in cultured cells, BMM were collected following LPS (0.1 µg/ml) stimulation. TTP was undetectable at time 0 but readily detectable after 2 h, reaching a peak of expression at about 4 h (Fig. 5A). Immunoreactive TTP in 500 µg of BMM protein was compared with only 50 µg of protein from RAW 264.7 cells stimulated with LPS for 1.5 and 2 h (Fig. 5A).

Similar induction kinetics were seen in MEF stimulated with 10% FCS (Fig. 5B). Again, TTP was undetectable in the serum-deprived cells at time 0, but accumulated dramatically to reach peak levels at ~2–3 h. These peak levels, detected with 200 µg of cellular protein per lane, were comparable to the levels seen in LPS-stimulated RAW 264.7 cells when only 10 µg of protein was loaded into the gel lane (Fig. 5B). Interestingly, there was a continued shift upward in the apparent molecular weight of the protein with still longer times of exposure to FCS (Fig. 5B), compatible with increasing phosphorylation. Protein was still readily detectable at 5–6 h.

To estimate the stability of the newly synthesized protein in these cells, the cells were stimulated for 2 h with 10% FCS and then treated with CHX (50 µM) for a further 4 h (Fig. 5B). Remarkably, there was very little apparent disappearance of the protein over 4 h following treatment with CHX (Fig. 5B), at concentrations that completely inhibited protein synthesis in this cell type (data not shown).

Because RAW 264.7 cells produced much more TTP than the other cell types we tested, we analyzed the induction kinetics in more detail in these cells. As shown in Fig. 5 (C and D), TTP

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**Fig. 2. Identification of endogenous TTP in mouse spleen.** A, 2 µg of protein from 293 cell extracts expressing HA-mTTP, and 5 mg of protein from 20,000 × g supernatants of spleen homogenate from WT and TTP KO mice, were separated by SDS-PAGE and transferred onto nitrocellulose membranes for Western blotting. The membrane was incubated in anti-MBP-mTTP serum (1:10,000) overnight and with GAR-HRP (1:10,000) for 1 h and exposed to x-ray film for 1 min. B, the 20,000 × g supernatant from mouse spleen was further centrifuged at 100,000 × g for 1 h to separate microsomal membranes (Membranes) from the cytosol (Soluble). Equal amounts of protein (1.7 mg) from the soluble and membrane fractions were used for Western blotting as above, along with MBP-mTTP (10 ng) from the amylose resin column and HA-mTTP in the transfected 293 cell extracts (1 µg of total soluble protein). In this case, the membrane was exposed to x-ray film for 10 min.
was undetectable in unstimulated cells and in cells stimulated with LPS for 15 and 30 min, using 50 μg of cytosolic protein and a relatively short autoradiographic exposure (Fig. 5, C and D). TTP could be detected in the 0.5-h samples if much more protein and/or much longer autoradiographic exposure were used (data not shown). TTP was detectable following induction for 45 min (Fig. 5C). At 1 h, TTP was detected as two major bands of apparent molecular weight 40–50,000, which stabilized by 2 h (Fig. 5, C and D). Significant amounts of immunoreactive TTP were still detectable in samples collected after 24 h of LPS induction (Fig. 5D). We also calculated TTP concentration in the stimulated RAW 264.7 cells with 0.1 μg/ml LPS for 2 h using known concentrations of the purified MBP-mTTP (1–100 ng). The immunoreactivity in 50 μg of RAW 264.7 cell 10,000 g supernatant was approximately the same as that of 10 ng (0.134 pmol) of the recombinant protein. This represents about 340 times the concentration in normal spleen, or ~0.5 μM.

The stability of TTP in RAW 264.7 cells was further evaluated following LPS stimulation for 2 h (0.1 μg/ml), followed by treatment with CHX (50 μM). No TTP was detected in RAW 264.7 cell extracts 1, 1.5, 2, 3, 4, or 5 h after both LPS and CHX were added to the culture medium at the same time, indicating that this concentration of CHX was effective at inhibiting protein synthesis under these experimental conditions (data not shown). Despite the inhibition of protein synthesis, there was only a modest decrease of TTP immunoreactivity over the next 4 h, although there was a continued shift to apparently greater molecular weight between 2 and 3 h after LPS, even in the presence of CHX (Fig. 5E). Similar slow rates of protein disappearance were observed in cells stimulated with LPS for 2.5 h followed by LPS “washout” and CHX treatment (data not shown).

Monomeric Nature of TTP in RAW 264.7 Cells—The 10,000 × g supernatant from RAW 264.7 cells stimulated with LPS (0.1 μg/ml) for 2 h was subjected to size exclusion chromatography following ammonium sulfate concentration. TTP could be detected in a range of column fractions, but the peak of TTP was detected in fraction #31 (Fig. 6A), which corresponded to a monomer size of about 40 kDa when compared with a standard curve (Fig. 6B). Small amounts of TTP were detected in earlier fractions (Fig. 6A), suggesting that a small proportion of the protein might exist in RAW 264.7 cells as oligomers or in complexes with other proteins under these conditions.

When LPS-stimulated RAW 264.7 cells were fractionated into cytosolic and nuclear fractions, most of the TTP was in the cytosolic fraction, with little if any associated with the nuclear fraction (Fig. 6C); there was no evidence of higher molecular weight oligomers or aggregates under these conditions. There was also some degree of cross-reactivity with the human TTP protein expressed in and purified from E. coli (Fig. 6C, lane 5). This figure also illustrates the extent of the inhibited SDS-PAGE migration caused by, presumably, phosphorylation in the RAW 264.7 cells. Human TTP has a predicted molecular weight of 34,086, and the protein purified from E. coli migrated as a single band at M_r ~36,000, as estimated with protein standards (lane 5), as we have shown previously (6). However, the mouse protein in LPS-stimulated RAW 264.7 cells, with a calculated molecular weight of 33,613, migrated as multiple bands of apparent molecular mass 40–50 kDa. The migration
of TTP was increased following dephosphorylation of mTTP in the RAW 264.7 cell 10,000 × g supernatant with CIAP treatment (Fig. 6D). Incubation with longer time or with more CIAP resulted in a faster migration of the protein, although the sizes of those dephosphorylated proteins were still larger than that of hTTP purified from E. coli cells (data not shown). This change in SDS-PAGE migration appears to be largely due to phosphorylation, because the multiband complex collapses into a single protein band of lower apparent molecular weight upon dephosphorylation with alkaline phosphatase (27).

**Immunostaining of TTP in Intact Cells and Tissues**—To further localize TTP in RAW 264.7 cells, they were treated with 0.1 μg/ml LPS for 3 h, followed by immunostaining with anti-MBP-mTTP (1:8,000 dilution). As shown in Fig. 7A, confocal microscopy revealed bright cytosolic immunofluorescence in LPS-treated RAW 264.7 cells, whereas minimal fluorescence was detected in controls treated in parallel with LPS induction and pre-immune serum; without LPS induction but with pre-immune serum. The cytosolic staining was in a vesicular pattern (Fig. 7A). There were occasional foci of nuclear staining, but these were seen also in the cells not treated with LPS; they were more prominent with immune serum than in the other two negative controls (Fig. 7A), suggesting that they may represent true foci of nuclear TTP. Serial sections of a single RAW 264.7 cell following LPS induction showed that the vesicular pattern of fluorescence was almost exclusively cytosolic, with little if any signal in the nucleus (Fig. 7B).

Consistent with the immunoblotting results, TTP was barely detectable under unstimulated conditions but peaked in the cytosol by 2–3 h following LPS stimulation (Fig. 7C). Significant immunostaining was still visible in the RAW 264.7 cells after 5 h, although the signal was somewhat decreased relative to the earlier time points (Fig. 7C). Although there was minimal detectable fluorescence in the unstimulated cells in Fig. 7 (A and C), we occasionally noticed increased fluorescence in the cytosol of cells undergoing division, even in the absence of LPS (Fig. 7D).

We also evaluated the use of the antibody in rat spleen, after the intraperitoneal injection of LPS (5 mg/kg). 2 h after the injection, there was prominent TTP staining in macrophages and stromal cells in the spleen periphery (Fig. 8A), whereas no specific staining was seen in spleen from a PBS-injected rat (C) or with preimmune serum staining of spleen from either an LPS-injected (B) or PBS-injected (D) rat. The light microscopic images in A–D were obtained and processed under identical conditions. Of interest was the negative staining of the white pulp in the center of Fig. 8A (arrow) (see “Discussion”). This immunostaining was seen with the use of Bouin’s fixed, paraffin-embedded sections, but not with spleens fixed in 4% paraformaldehyde and processed for frozen sections (data not shown).

**DISCUSSION**

Despite more than 13 years of information about the transcriptional induction of TTP in response to a variety of growth factors, cytokines, and mitogens, relatively little is known about the behavior of the TTP protein itself under similar circumstances (1). This lack of information is due in part to difficulties in expressing the recombinant protein, making preparation of high affinity antibodies more difficult (6). However, the most difficult problem to overcome has been the apparent extraordinary scarcity of the protein in most cells and tissues. We have begun to approach some of these issues by developing a high titer antibody to the recombinant mouse TTP protein expressed in E. coli as a MBP fusion protein.

One of the fundamental questions we hoped to address with this new antiserum was whether TTP protein was relatively stable in cells after its induction by mitogens and cytokines. This question arose because of the archetypal immediate-early response gene characteristics of the TTP mRNA after its induction by insulin, serum, or other mitogens in cultured fibroblasts.
For example, in mouse 3T3-L1 adipocytes, TTP mRNA was essentially undetectable by Northern blot in the basal, serum-deprived G0 state. However, within 10 min of exposure to insulin, there was a detectable level of mature TTP transcript, and this continued to accumulate for the first 45 min, after which there was a rapid decline in transcript levels to near basal concentrations by 120 min (13). Similar induction and turnover kinetics were observed in what is believed to be a physiologically relevant cell type and response, i.e. the induction in macrophages by agents such as LPS or TNF (7). In both situations, the rapid and dramatic induction of transcription was followed by a similarly rapid shut-off of transcription accompanied by mRNA lability, such that steady-state levels of mRNA had returned to near-basal levels despite the continued presence of the inducing stimulus (1).

This rapid and transient transcript response is characteristic of prototypical immediate early response genes such as c-fos. However, this type of response did not fit with the proposed physiological function of the TTP protein, i.e. to bind to and destabilize mRNAs such as those encoding TNF and GM-CSF (1). For example, TNF mRNA is rapidly and dramatically induced by LPS in macrophages, followed by the production of secreted TNF (7); how can we reconcile the rapid but transient TTP transcript response with the presumed continued requirement for TTP protein to cause degradation of the TNF transcript and reversal of TNF secretion?

In the present study, we addressed this apparent discrepancy by evaluating the induction patterns of TTP protein and its stability in cultured MEF after stimulation with serum, and in cultured mouse RAW 264.7 macrophages after stimulation by LPS. In the latter case, TTP protein was readily detectable as multiple bands on Western blots of a crude cytoplasmic fraction from the cells after induction, although the protein was barely detectable in the unstimulated cells, as noted previously (28). These multiple bands were shown previously to be multiple phosphorylated species, because treatment of the protein in extracts from 293 cells with alkaline phosphatase caused collapse of the multiple bands into a single, presumably dephosphorylated species (27). In LPS-stimulated RAW 264.7 cells, TTP protein accumulated to reach near steady-state levels after 90–120 min; remarkably, these high levels of protein remained nearly constant for many hours thereafter, and were still readily detectable after 24 h. In keeping with this pattern, treatment of the cells with CHX 2 h after LPS stimulation, at concentrations shown to completely inhibit protein synthesis in these cells, revealed a very stable protein, with minimal decline
in protein levels observed for several hours, until generalized CHX toxicity began to develop. Similar patterns were observed in serum-treated mouse fibroblasts, again with the formation of a very stable protein. Taken together, these data make physiological sense, in that the continued presence of the newly synthesized protein would be expected to continue to exert its negative regulatory effects on ARE-containing transcripts for several hours after the initial stimulation.

Another interesting aspect of this protein induction pattern is the gradual increase in apparent phosphorylation that accompanied the increase in steady-state protein levels. Both maximal phosphorylation, as indicated by maximal increase in apparent molecular weight on SDS gels, and the steady-state protein level were achieved by 4 h after stimulation in both macrophages and fibroblasts. This unusual case, in which previously synthesized protein is phosphorylated within a few minutes by an activated protein kinase. In this unusual case, phosphorylation and biosynthesis appeared to occur in parallel, raising interesting questions about the types of protein kinases involved, their regulation, and their effects on the protein. To date, sites for the broad family of mitogen-activated protein kinases have been identified in the protein (18), as well as sites for the MKK2 kinase that appear to be involved in binding of the protein to 14-3-3 protein (29, 30). Nonetheless, many more phosphorylation sites remain to be identified on this protein. It is interesting to speculate that the increase in phosphorylation that accompanied the increased biosynthesis of the protein might be involved in conferring stability to the protein, as noted previously for p53, for example (31, 32). Obviously, other regulatory possibilities of this phosphorylation exist, including the regulation of the nuclear to cytoplasmic shuttling of TTP, its binding affinity for its RNA targets, its association with other proteins, and others.
The studies described here are in general agreement with previous studies of TTP expression and subcellular localization. For example, Taylor et al. (18) used a mouse amino-terminal anti-peptide antibody and immunoprecipitation to demonstrate the induction of TTP in mouse fibroblasts and the decreased gel mobility upon cell stimulation with various mitogens, due in part to phosphorylation by one or more mitogen-activated protein kinases. They also used the same antiserum to show that TTP in stably transfected serum-deprived fibroblasts was mostly in the nucleus but translocated to the cytosol within 5 min of serum stimulation (33). Carballo et al. (7) found a massive increase of TTP in the cytosol of primary mouse macrophages 4 h after LPS or TNF stimulation, using radioactive labeling and immunoprecipitation with the same anti-peptide antiserum. Lai et al. (8) were able to immunoprecipitate mouse macrophage TTP cross-linked to radiolabeled TNF RNA and found that most overexpressed TTP was present in the cytosol of 293 cells. Rigby and colleagues (34) used an affinity-purified polyclonal antibody directed against a carboxyl-terminal peptide from human TTP to show that most immunoreactive TTP was in the cytosol of LPS-stimulated THP-1 cells and could be induced rapidly and transiently in those cells after LPS stimulation (35). Interestingly, their data from THP-1 cells suggested a more transient response of the TTP protein to LPS stimulation than seen in the mouse cells described here. The protein turnover rate was not commented on in those studies, nor was the apparent size change upon stimulation noted, probably because most of their protein assays involved fluorescence-activated cell sorting.

In the present study, TTP was essentially undetectable in unstimulated RAW 264.7 macrophages, although slightly higher levels of punctate nuclear staining were observed in these cells stained in parallel with the immune serum compared with pre-immune serum. This result is compatible with a previous study in serum-deprived cultured fibroblasts stably expressing TTP driven by a metallothionein promoter, in which

![Fig. 7. Confocal microscopy detection of TTP in RAW 264.7 cells. A, confocal microscopy of RAW 264.7 cells. Cells were treated with either LPS (0.1 𝜇g/ml) or PBS for 3 h, then fixed and stained with either the anti-MBP-mTTP serum (I) or preimmune serum (PI) (1:8,000 dilution). B, cytosolic localization of TTP in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (0.1 𝜇g/ml) for 2 h and stained with anti-MBP-mTTP serum (1:10,000 dilution) as described in A. C, time course of TTP induction in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (0.1 𝜇g/ml) for 0, 2, 3, and 5 h as indicated and stained with anti-MBP-mTTP serum (1:10,000 dilution) as described in A. D, TTP immunostaining during cell division in RAW 264.7 cells. Unstimulated RAW 264.7 cells were stained with the anti-MBP-mTTP serum (1:10,000 dilution), as described in A. Immunoreactive TTP was visible in the cytoplasm of the dividing cell indicated by the arrowheads in D, panel 1, whereas no cytoplasmic staining was visible in the other cells in the field. A light microscopic image of the same field of cells is also shown (D, panel 2).]
unstimulated cells expressed predominantly nuclear TTP, but the protein had shifted into the cytosol within 5 min of serum stimulation (33). We also noted in the current study that there was detectable cytoplasmic expression in dividing cells, even without LPS stimulation. Within an hour or so of LPS staining in the cultured macrophages, clear-cut cytoplasmic staining was observed, which remained readily detectable in an interesting vesicular pattern for several hours. Cellular fractionation of similarly treated cells confirmed the cytoplasmic localization of almost all immunoreactivity in the LPS-treated cells. The nature of the vesicular pattern observed in the cytoplasm is not clear, but most immunoreactivity did not sediment with a membrane fraction centrifuged at 100,000 g for 1 h. Co-staining with markers of other cellular organelles previously associated with TTP, such as exosomes (36) and stress granules (37), as well as other organelles will be of interest in this regard.

We also used this antiserum to probe the expression of TTP protein in normal tissues of adult animals. This required extraordinarily large amounts of protein to be loaded into the gel wells so that Western blots could be performed, with comparable samples from TTP KO mice used to ensure the specificity of the observed bands. When this was done, protein was detectable as multiple bands in similar tissues to those previously characterized as expressing the most mRNA by Northern blotting; these include spleen, thymus, lung, and liver, with lower or essentially undetectable expression in other tissues (13). One somewhat surprising finding was the relatively high level expression in large intestine, with much lower levels in small intestine; this is in contrast to previous Northern blot data, in which high level mRNA expression was observed in small intestine.2 This is also interesting given the fact that the TTP KO mice on a C56Bl6 background do not seem to develop a Crohn’s-like colitis, in contrast to mice in which the ARE of the TNF transcript has been removed, resulting in a stable transcript and TNF overexpression (38). Identification of the cell types expressing TTP under normal circumstances and in models of experimental colitis will be of interest in this regard.

In a very recent report in which a commercial antibody was used, the authors described a very different pattern of TTP tissue distribution in the mouse (39). For example, they detected the highest level of expression in liver, with significant expression in testis and ovary, with TTP being represented as a single sharp band of M, 43,000; in contrast, we could not detect immunoreactivity in the testis. In addition, they reported minimal to undetectable expression in spleen, thymus, lung, and intestine, in contrast to our results showing readily

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2 G. A. Taylor and P.J. Blackshear, unpublished data.

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Fig. 8. TTP immunostaining in rat spleen. Rats were injected intraperitoneally with LPS (5 mg/kg; A and B) or PBS (5 ml/kg, C and D), and spleens were removed and used for immunostaining of TTP with either the anti-MBP-mTTP serum (I) or pre-immune serum (PI) as described under “Materials and Methods.” The reddish brown staining in A represents TTP immunoreactivity, whereas none was seen in the other three panels. The arrows in A point to the white pulp of the spleen, which is unstained by the TTP antibody. See the text for other details.
detectable expression in those tissues. However, certain critical controls were omitted from their report, including the use of pre-immune serum, overexpressing cells as a positive control, and tissues from KO mice as negative controls, casting some doubt on the reliability of their expression data.

We used these Western blot data to calculate an approximate intracellular concentration of TTP in spleen of normal adult mouse and in LPS-stimulated RAW 264.7 cells. This was done by comparing the expression levels of a known amount of recombinant protein to that contained in a known amount of spleen cytosol and RAW 264.7 cells. This calculation revealed an overall concentration in spleen of about 1.5 nM, and in maximally stimulated RAW 264.7 cells of about 0.5 μM. The extraordinarily low value in spleen may account for previous difficulties in determining protein levels in normal tissues and high background of nonspecific bands on Western blots reported here. The dramatic stimulation of TTP in RAW 264.7 cells by LPS may explain why TTP could be easily detected in the saline-injected rat spleen. Obviously, there are differences between normal circumstances or 2 h after injection of LPS.

We used these Western blot data to calculate an approximate concentration by comparing the expression levels of a known amount of purified recombinant protein to that contained in a known amount of endogenous protein in RAW 264.7 cells. When TTP is overexpressed or underphosphorylated in RAW 264.7 cells, it is common to observe apparent dimers or oligomers of TTP, or possibly hetero-oligomers with putative TTP-interacting proteins. Nonetheless, there was no doubt on the reliability of their expression data.

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Immunological Characterization of Tristetraprolin as a Low Abundance, Inducible, Stable Cytosolic Protein
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