

Review

The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis

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Abstract

Tristetraprolin (TTP) is the best-studied member of a small family of three proteins in humans that is characterized by a tandem CCCH zinc finger (TZF) domain with highly conserved sequences and spacing. Although initially discovered as a gene that could be induced rapidly and transiently by the stimulation of fibroblasts with growth factors and mitogens, it is now known that TTP can bind to AU-rich elements in mRNA, leading to the removal of the poly(A) tail from that mRNA and increased rates of mRNA turnover. This activity was discovered after TTP-deficient mice were created and found to have a systemic inflammatory syndrome with severe polyarticular arthritis and autoimmunity, as well as medullary and extramedullary myeloid hyperplasia. The syndrome seemed to be due predominantly to excess circulating tumor necrosis factor- α (TNF- α), resulting from the increased stability of the TNF- α mRNA and subsequent higher rates of secretion of the cytokine. The myeloid hyperplasia might be due in part to increased stability of granulocyte-macrophage colony-stimulating factor (GM-CSF). This review highlights briefly the characteristics of the TTP-deficiency syndrome in mice and its possible genetic modifiers, as well as recent data on the characteristics of the TTP-binding site in the TNF- α and GM-CSF mRNAs. Recent structural data on the characteristics of the complex between RNA and one of the TTP-related proteins are reviewed, and used to model the TTP-RNA binding complex. We review the current knowledge of TTP sequence variants in humans and discuss the possible contributions of the TTP-related proteins in mouse physiology and in human monocytes. The TTP pathway of TNF- α and GM-CSF mRNA degradation is a possible novel target for anti-TNF- α therapies for rheumatoid arthritis, and also for other conditions proven to respond to anti-TNF- α therapy.

Keywords AU-rich elements, deadenylation, granulocyte-macrophage colony-stimulating factor, mRNA turnover, rheumatoid arthritis, tumor necrosis factor

Introduction

The transcript encoding the tandem CCCH zinc finger (TZF) protein tristetraprolin (TTP) was discovered nearly 15 years ago in screens for genes that were rapidly turned on by exposure of cultured fibroblasts to insulin, serum, or tumor-promoting phorbol esters [1–4]. The transcript was

rapidly but transiently induced by these and other stimuli. Because of the immediate-early response gene characteristics of this induction, the presence of two nearly identical putative zinc fingers in the predicted protein sequence, and early data indicating nuclear localization, TTP was thought likely to be a transcription factor.

ARE = AU-rich element; EGP = Environmental Genome Project; EST = expressed sequence tag; GM-CSF = granulocyte-macrophage colony-stimulating factor; KO = knockout; LPS = lipopolysaccharide; MAP = mitogen-activated protein; NMR = nuclear magnetic resonance; PARN = poly(A) exonuclease; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; SNP = single-nucleotide polymorphism; TNF- α = tumor necrosis factor- α ; TNF- α R = tumor necrosis factor receptor; TTP = tristetraprolin; TZF = tandem CCCH zinc finger; UTR = untranslated region; WT = wild-type; ZFP36L1 = zinc finger protein 36-like 1; ZFP36L2 = zinc finger protein 36-like 2.

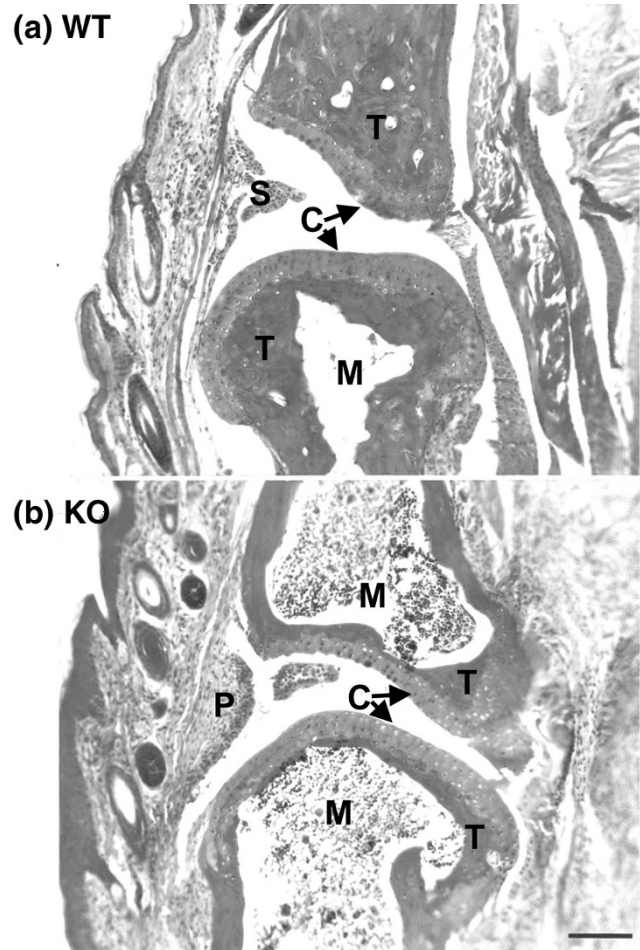
During the succeeding several years, the protein was found to be translocated from the nucleus to the cytosol, and also phosphorylated, by the same agents that stimulated the accumulation of its mRNA [5,6]. Its gene, labeled *ZFP36* in humans (for zinc finger protein 36 [7]), was found to be highly regulated by known and novel transcription-factor-binding sites in both the classical promoter domain and in the single intron [8,9]. Two other predicted proteins containing the characteristic TZFs were also identified during this time; their official gene names are *ZFP36L1* and *ZFP36L2* (for zinc finger protein 36-like 1 and 2) in humans, although the encoded proteins have numerous aliases (see [10] for a brief recent review).

Despite this progress, essentially nothing was known about the function of this small family of proteins with the unusual, hypothetical zinc fingers until the development of the TTP knockout (KO) mouse [11]. These animals appeared normal at birth but soon developed a phenotype that included an erosive, polyarticular arthritis. A key observation was that this arthritis, as well as most other aspects of the phenotype, could be prevented by treating young mice with weekly injections of an antibody against tumor necrosis factor- α (TNF- α). This observation, and subsequent experiments on the role of TTP in TNF- α regulation, led to speculation that TTP might be involved in some aspects of human arthritis. In this brief review we will discuss the following: first, the characteristics of the TTP KO phenotype; second, recent experiments on genetic modifiers of this phenotype; third, recent data on the nature of the interaction between TTP and the TNF- α transcript; fourth, the current status of known TTP sequence variants in humans; and fifth, potential roles of the TTP-related proteins *ZFP36L1* and *ZFP36L2*. This review is not meant to be comprehensive but instead will focus on our personal experiences with this family of proteins over the past 14 years.

Characteristics of the TTP KO phenotype

As noted above, there seemed to be minimal, if any, excess prenatal mortality of TTP KO mice [11]. However, the pups soon exhibited a slow-growth phenotype, which ranged from minimal to quite severe. This was associated with marked secondary loss of adipose tissue in all depots, and this cachexia was one of the key findings that led us to suspect a link with TNF- α , formerly known as 'cachectin'. Other aspects of the phenotype included patchy alopecia and dermatitis, conjunctivitis, and the 'kangaroo' hunched posture that is characteristic of many inflammatory syndromes in mice. However, one of the most striking aspects of the external phenotype was the apparent symmetrical arthritis, manifested by reddened, swollen paw joints. This had been seen previously in other TNF- α excess syndromes [12,13] and was a second major clue that effective TNF- α excess might have a role in the disease pathogenesis.

Figure 1

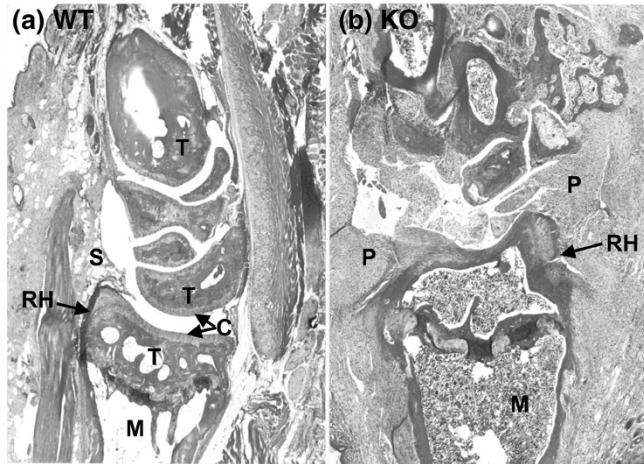


Interphalangeal joints in wild-type (a) and tristetraprolin knockout (b) mice. Shown are matching joints from littermate mice at about 7 months of age, stained with hematoxylin and eosin. C, articular cartilage; M, marrow; P, pannus; S, synovium; T, trabecular bone. Scale bar, 0.5 mm. Modified from [11].

Histologically, the arthritis was severe and erosive. Figure 1 compares interphalangeal joints from littermate wild-type (WT) and TTP KO mice at about 7 months of age. In this comparison there is a clear increase in the size and inflammatory nature of the synovium, which resembles an early pannus; however, the articular cartilage appears relatively normal in this joint. However, there was a very striking increase in the cellularity of the marrow compartment in the KO mice, in which the marrow cavities were packed with normal-appearing granulocytes, with accompanying erosion of the surrounding bone.

In a larger joint, in this case the 'wrist' of the mice, the changes were more marked (Figs 2 and 3). The ordinarily delicate synovium had been transformed into an exuberant pannus, which seemed to be eroding the articular cartilage as well as underlying bone. The smaller bones of

Figure 2



Radial head histology in wild-type (a) and tristetraprolin knockout (b) mice. This low-power view is of radial head joints from littermate mice at about 7 months of age, with the radial head (RH) indicated. Other abbreviations are as in the legend to Fig. 1. Modified from [11].

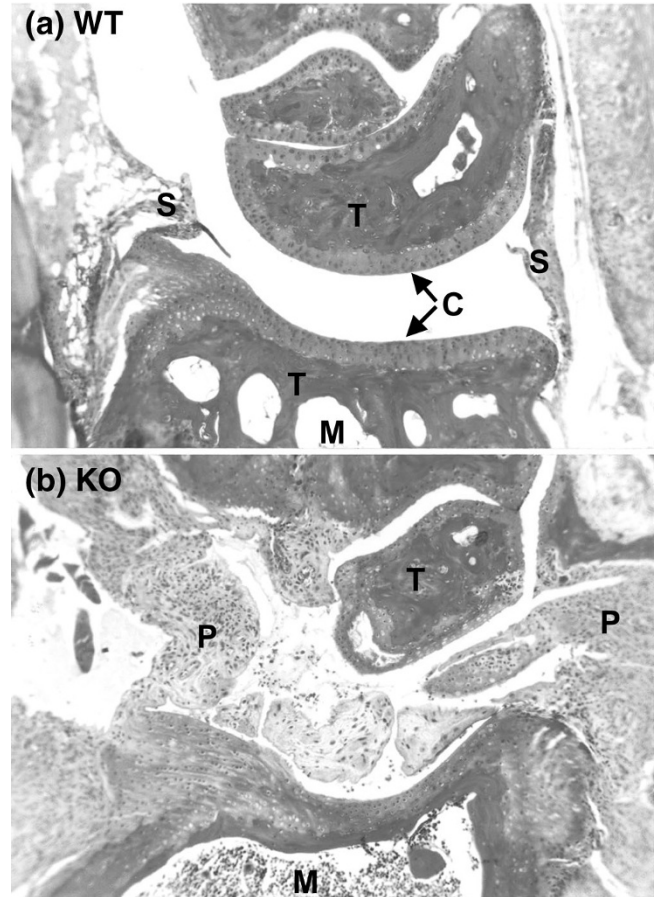
the metacarpals were often destroyed, and loss of digits was not uncommon. As in the smaller joints described above, there was marked proliferation of marrow granulocytes, essentially all of which were Gr-1⁺, again with internal erosion of both trabecular and cortical bone.

This histological picture was similar to that seen in other models of TNF- α excess, although in most cases the marrow granulocytosis was either not found or not commented on [12–14]. One exception was the syndrome induced by periodic injections of TNF- α into mice, in which the authors reported a marked increase in marrow granulocytes [15].

Other pathological characteristics included granulocyte infiltration in the skin, accompanying the loss of subcutaneous fat; and foci of granulocytes in the liver, spleen, lymph nodes and other extramedullary sites. This was accompanied by marked splenomegaly and lymphadenopathy in most cases. In the blood, there was an approximately twofold increase in total white blood cell count; most of the increase could be accounted for by nearly a fourfold increase in Gr-1⁺ granulocytes, a threefold increase in F4/80⁺ macrophages, and increases in PK135⁺ natural killer cells. There were decreases in both B and T lymphocytes. Erythrocyte and platelet counts were essentially normal. There were also marked increases in myeloid progenitors in spleen and peripheral blood, but not in bone marrow, in the KO mice.

Serologically, autoimmunity was present in the form of high titers of antinuclear antibodies that stained in a

Figure 3



Higher-power view of the radial head histology for the same littermate wild-type (a) and tristetraprolin knockout (b) mice as those shown in Fig. 2, with the radial head at the bottom of each panel. Abbreviations are as in the legend to Fig. 1. Modified from [11].

homogeneous pattern, as well as antibodies against both double-stranded and single-stranded DNA. However, rheumatoid factor titers were normal (both IgG and IgM), as were anti-Sm antibody titers. There were some histological abnormalities of the kidneys, but no increase in IgG or IgM staining of the glomeruli was noted, and no proteinuria or azotemia was found in the KO mice.

Because many aspects of the mouse phenotype resembled previous models of chronic, systemic TNF- α excess, we performed an experiment in which hamster anti-mouse TNF- α monoclonal antibodies were administered to TTP KO mice as soon as they could be genotyped after birth [11]. Remarkably, these injections prevented the development of essentially the entire TTP deficiency phenotype. Specifically, the cutaneous, joint, adipose tissue, and hematological abnormalities were prevented. This led to the conclusion that most if not all of the abnormalities noted in the KO mice were due to

chronic 'effective elevation' of circulating TNF- α . This was strongly supported by later studies in which the TTP KO mice were interbred with mice lacking one or both types of TNF- α receptor (see below).

The next studies were aimed at elucidating the mechanism of this apparent TNF- α elevation. An important clue was that the phenotype could be transferred by whole bone marrow transplantation into *Rag2*^{-/-} recipient mice, but only after a rather long latent period, suggesting that the phenotype was not transferred with lymphocyte progenitors but instead by more slowly reconstituting cells such as those of the macrophage/monocyte lineage [16]. We then demonstrated that KO macrophages derived from several sources, including fetal liver, adult bone marrow, and peritoneal cavity, all released considerably more TNF- α than did macrophages from their WT littermates [16]. Critically, this was associated with an increase in the steady-state levels of TNF- α mRNA in the KO cells, implying either an increase in TNF- α gene transcription or a decrease in TNF- α mRNA turnover rates, or both.

Subsequent studies in bone marrow-derived macrophages demonstrated that there was a marked decrease in TNF- α mRNA turnover rate in KO cells stimulated with lipopolysaccharide (LPS) and then treated with actinomycin D to inhibit transcription, implicating TTP in the process of TNF- α mRNA turnover [17]. To assess the possibility that TTP could be causing this effect by first binding directly to the transcript, we performed direct RNA binding studies with ultraviolet crosslinking of protein to RNA followed by immunoprecipitation with anti-TTP antibodies, and RNA gel-shift analyses. These studies revealed that TTP did indeed bind directly to the TNF- α transcript, in an AU-rich region long known to be an instability agent for this relatively unstable message, and that the TZF domain of TTP was the RNA-binding domain of the protein [17].

These studies showed that TTP was an AU-rich element (ARE)-binding protein that destabilized its target mRNA, in this case the mRNA encoding TNF- α . This led us to postulate a TTP-TNF- α feedback loop, in which both TNF- α and LPS stimulated the transcription of both the TNF- α and TTP genes, with the latter protein product feeding back to bind to the TNF- α mRNA and destabilizing it, thus potentially reversing the effects of the initial stimulus and the subsequent positive feedback effect that TNF- α has on its own transcription [17].

The mechanism of the effect of TTP remains to be determined, but an important clue came from our evaluation of a second mRNA containing a TNF- α -like ARE: that encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) [18]. TTP could bind to this element as well as to the TNF- α ARE. In addition, the

transcript from KO bone marrow-derived stromal cells was essentially completely stable after treatment of the cells with actinomycin D, whereas the transcript was unstable in the corresponding WT cells. This indicated that the GM-CSF mRNA was likely to be another physiological target for TTP. Most importantly, however, the pattern of GM-CSF transcripts revealed by northern blotting was extremely informative. In total cellular RNA from the WT stromal cells, the GM-CSF mRNA existed as two species of approximately equal abundance, differing in size by about 200 bases. This size difference was shown to be due to the presence or absence of a poly(A) tail. Strikingly, almost all of the stable transcripts in the KO cells were in the fully polyadenylated form. This meant that the absence of TTP led to a failure of deadenylation, or removal of the poly(A) tail, from the GM-CSF transcript. This is thought to be the first step in vertebrate mRNA degradation [19–21], and it seemed likely that the inability of the GM-CSF mRNA to be deadenylated in the KO cells led directly to the increase in its stability. Conversely, it meant that TTP could be viewed as an mRNA-binding protein whose destabilizing abilities were likely to be due to its ability to promote the process of transcript deadenylation.

In subsequent studies, we developed both cell transfection assays and cell-free deadenylation assays to show that, indeed, TTP could promote the deadenylation and breakdown of mRNAs containing the characteristic ARE-binding sites [22,23]. This ability of TTP to promote deadenylation was enhanced by increasing the concentrations of poly(A) exonuclease (PARN), either in intact cells or in cell-free assays, suggesting that the two proteins could act synergistically to promote the deadenylation of a relatively small number of transcripts containing characteristic ARE TTP-binding sites [23]. The exact molecular nature of this interaction is the subject of continuing studies, but it should lead us to a molecular understanding of how TTP promotes the deadenylation and destabilization of a specific set of target proteins.

Now that the principal target of TTP relevant to the TTP-deficiency phenotype is known to be the TNF- α mRNA ARE, it is instructive to compare the TTP KO phenotype with others in the literature that have involved TNF- α specifically. In the first such model developed, Kollias and colleagues created transgenic mice in which human TNF- α was overexpressed in T lymphocytes [14]. These animals developed many of the same phenotypes as the TTP KO mice, including wasting and cachexia, premature death, and universal inflammatory arthritis, all of which could be prevented by the administration of an antibody against TNF- α . Another directly relevant model was that created by specifically removing the TNF- α mRNA ARE (Δ ARE) from the endogenous mouse TNF- α gene, resulting in a markedly more stable TNF- α mRNA [13]. As expected, this led to the chronic oversecretion of TNF- α

due to increased accumulation of the stabilized mRNA, in a manner analogous to that in the TTP KO mice.

In general, the phenotype of this mouse was considerably more severe than the TTP KO phenotype. For example, although these mice were born alive, the KO animals rapidly developed a marked slow-growth phenotype and died between 5 and 12 weeks of age. Pathologically, these mice were characterized by severe erosive arthritis and inflammatory bowel disease, but inflammation in other tissues was minimal. They also exhibited thymic atrophy and blurring of thymic cortical and medullary boundaries, also characteristic of the TTP KO phenotype. The arthritis developed as early as 12 days of age, and resembled the TTP KO arthritis in general character; however, the Δ ARE mice exhibited elevated levels of mouse IgG and IgM rheumatoid factor, not seen in the TTP KO mice. The inflammatory bowel disease was severe and universal and affected both the colon and the terminal ileum in some cases. The hemizygous Δ ARE mice had a less severe phenotype, but most aspects of the same syndrome were seen with a delayed onset relative to the homozygous mice [13].

In a striking difference from the TTP KO mice, both the Δ ARE homozygous and hemizygous mice developed a severe form of Crohn's-like colitis, a syndrome that has not been seen in the TTP KO mice so far [13]. We have recently shown that TTP protein is relatively highly expressed in normal mouse colon [24], and its absence from this tissue might be expected to have deleterious effects on this organ. Much remains to be determined about the specific cell types that express TTP in this tissue, which may not be directly relevant to the development of the colitis syndrome. Nonetheless, this remains an interesting difference between the Δ ARE mice and the TTP KO mice that is yet to be explained.

Genetic modifiers of the TTP-deficiency syndrome

We and others have evaluated the TTP-deficiency phenotype further by interbreeding the mice with other potentially informative genotypes. To confirm the data from the TNF- α antibody injection experiments, we interbred the TTP-deficient mice with mice deficient in either or both TNF- α receptors 1 and 2 (TNF- α R1 and TNF- α R2) [25]. As predicted from the antibody experiments, TTP KO mice that were also deficient in both types of TNF- α receptor seemed essentially normal, with none of the cutaneous or joint manifestations of the TTP-deficiency syndrome. Most of the protective effect was due to the TNF- α R1 deficiency; the TTP/TNF- α R1 double KO mice were also essentially normal. Interestingly, the TTP/TNF- α R2 double KO mice were even more severely affected than the parental TTP KO strain, with very severe arthritis and growth retardation in early life leading to premature death

in most cases. This supported the concept, developed in other studies, that TNF- α acting through TNF- α R2 can exert a protective effect on the TNF- α -induced inflammatory response in some situations. In any case, these studies confirmed the findings of the TNF- α antibody injection study; it seemed clear that most aspects of the TTP-deficiency phenotype were thus due to chronic, elevated levels of TNF- α .

However, observation of the normal-appearing TTP/TNF- α R1/TNF- α R2 triple KO mice for longer than about 1 year revealed that they were not quite normal. As these animals aged, there was the development of myeloid hyperplasia, particularly intramedullary, which eventually resembled that seen in the original TTP KO mice [25]. This finding, coupled with the known effect of TTP deficiency to stabilize the GM-CSF mRNA and lead to enhanced secretion of this cytokine [18], suggested the possibility that the myeloid hyperplasia seen in the aging triple KO mice might be due to chronic increases in the concentration of either circulating or local GM-CSF. We are currently attempting to address this possibility by interbreeding the TTP KO mice, and the TTP/TNF- α R1/TNF- α R2 triple KO mice, with mice deficient in GM-CSF [26]. The first mice of these genotypes are approaching an age at which they can be analyzed, and should yield valuable information: first, the extent to which the myeloid hyperplasia of the original TTP KO mice was due to GM-CSF; second, the extent to which the late-onset myeloid hyperplasia of the triple KO mice is due to GM-CSF; and third, if the myeloid hyperplasia is prevented, these 'quadruple KO' mice might possibly yield valuable insights into any remaining physiologically relevant TTP target transcripts.

Besides interbreeding with TNF- α R1, TNF- α R2 and GM-CSF KO mice, several studies have been performed with other potentially informative genetic backgrounds. These studies are arduous because of the marked subfertility of the TTP KO mice, necessitating that the line be maintained by hemizygotes. Recently, for example, Phillips and colleagues [27] generated mice deficient in both TTP and another TNF- α synthesis regulator, TIA-1. TIA-1 is thought to inhibit TNF- α production primarily by interfering with the translation of existing transcripts. As expected, the TTP/TIA-1 double KO mice developed more severe arthritis than either genotype alone. An unexpected result of this study was that macrophages derived from the TTP/TIA-1 double KO mice secreted less TNF- α protein than cells from either single KO alone, leading the authors to speculate that the TNF- α secretory apparatus might somehow be interfered with in these macrophages. They also found that bone marrow cells from the TTP KO mice, particularly in combination with the TIA-1 KO, secreted considerable TNF- α in response to LPS, in contrast to the minimal secretion from marrow cells from either WT mice

or TIA-1 KO mice. This enhanced secretion was also seen with isolated neutrophils, which accumulate markedly in the marrow and elsewhere in the TTP KO mice. The authors speculated that these neutrophils might represent the primary source of 'arthritogenic' TNF- α in this situation.

Other interbreeding experiments are either under way or have not yet been published. For example, J Rivera-Nieves has crossed the TTP-deficient mice with the TNF- α Δ ARE mice alluded to above (personal communication). This is a potentially interesting cross because the TNF- α Δ ARE mice develop a severe, Crohn's-like colitis, whereas the TTP-deficient mice have not been observed to do so, at least on the C57Bl6 background. Another interesting cross is the TTP-deficient mice interbred with a mouse in which TNF- α cannot be secreted because its biosynthetic cleavage is prevented, leading to the accumulation of cell-associated TNF- α but an absence of secreted TNF- α (G Kollias, personal communication). A third current study involves crossing the TTP KO mice with mice deficient in the p38 mitogen-activated protein (MAP) kinase target MK2, which seems to be involved in TNF- α mRNA translation and TTP post-translational modification (see below; M Gaestel, personal communication). Other studies with potentially interesting genetic modifiers are continuing, and the next several years should yield several interesting insights into regulatory molecules and pathways. In addition to these crosses into specific knockout mice, we are crossing the TTP deficiency genotype into other genetic backgrounds in the hope of identifying other modifiers.

Recent data on the TTP-binding site in the TNF- α and GM-CSF mRNAs

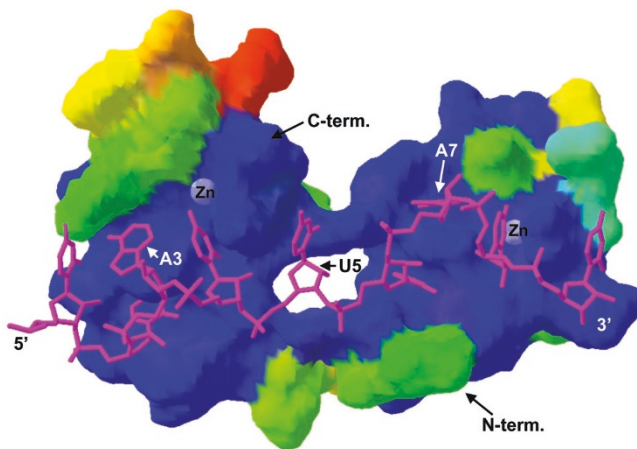
Since the pioneering work of Shaw and Kamen [28], it has been clear that certain AREs in mRNAs can confer instability on those transcripts. These have been categorized more recently into classes based on the presence or absence of AU substructures, such as the AUUUA pentamer. The TNF- α and GM-CSF AREs, so far the only definite physiological targets for TTP, fall into the type 2 ARE of Chen and colleagues [29], or the type 2a of Willusz and colleagues [21], in which there are multiple copies of tandem and overlapping AUUUA pentamers.

Several recent studies have characterized a more specific TTP-binding site. In the first, Worthington and colleagues used the SELEX procedure to identify the nonamer 5'-UUAUUUAUU-3' as the optimal site from a random collection of oligonucleotides [30]. Using a synthetic TZF domain peptide derived from the human TTP, known as TTP73, our group independently showed that this same nonamer sequence was optimal for TTP binding on gel-shift analysis, which could be accomplished with binding affinities in the low nanomolar range [31]. In a heteronuclear single-quantum coherence nuclear

magnetic resonance (NMR) analysis, we found that binding of the TTP peptide to target RNAs caused a major conformational shift in the resonances of the first zinc finger in the peptide, while simultaneously permitting the demonstration of second zinc finger resonances that had been apparently unstructured in the absence of RNA. Using progressive shortening of a longer, TNF- α ARE-based mRNA target, we found that the characteristic NMR resonance shift occurred in an identical manner down to the 5'-UUAUUUAUU-3' nonamer; however, loss of a single base in an octamer showed a deterioration of the NMR resonance pattern, and still shorter oligonucleotides were ineffective. These data suggested that the nonamer 5'-UUAUUUAUU-3' was the minimal complete binding site for the TTP peptide. This study also demonstrated that in longer AREs containing several repeats of this sequence, as are found in the TNF- α and GM-CSF AREs, several tandem molecules of the TTP73 peptide could bind to a single RNA strand, leaving open the possibility of multiple occupancy of the longer ARE by mature TTP protein molecules. The effect of these multiple tandem nonamer repeats on the effectiveness of TTP in promoting mRNA deadenylation is the subject of a continuing study.

Very recently, Hudson and colleagues determined the structure of the analogous TZF domain peptide from the TTP-related protein ZFP36L2 (TIS11D) in complex with this RNA nonamer [32]. This novel structure confirmed the conformational change in the peptide after RNA binding, and revealed many interesting aspects of the interaction between the TZF domain and RNA. For example, each of the two zinc fingers formed very similar structures, each of which contacted an RNA 'half-site' 5'-UAUU-3', with the binding apparently mediated by electrostatic and hydrogen-bonding interactions. The binding is further influenced by 'stacking' of the conserved aromatic amino acid side chains with the RNA bases. The 'lead-in' motifs R(K)YKTEL of each finger also participate through hydrogen-bonding interactions with the 5'-most bases on each half site. Importantly for future informatics analyses, the 5'-most U of the 5'-UUAUUUAUU-3' nonamer was disordered in the structure, which might allow base substitutions at this position in physiologically relevant RNA-binding sites, as well as less conservation in the protein sequence at the protein face near this base. The structure of this RNA-binding domain was thought to be unlike any previously published structure.

We have taken the coordinates of the ZFP36L2 TZF domain complex and modeled the structure of the interaction of the human TTP TZF peptide with the RNA nonamer, using the Swiss-Model programs [33,34]. A surface depiction of this model is shown in Fig. 4. It should be emphasized that this is a model based on the published ZFP36L2 TZF domain structure [32], but the two sequences are so closely related that it seems likely

Figure 4

Proposed structure of the human tristetraprolin (TTP) tandem zinc finger domain in complex with the TTP-binding site 5'-UUAUUUAUU-3'. This proposed structure was modeled on the original nuclear magnetic resonance structure described by [32], using their pdb coordinates and the Swiss-Model program. The RNA oligonucleotide is shown in magenta, with the 5' and 3' ends indicated, along with the key residues A3, U5, and A7. The peptide is shown as a surface structure, with the buried zinc residues highlighted and the amino-terminal (N-term.) and carboxy-terminal (C-term.) ends of the peptide shown by arrows. The dark blue residues represent amino acids that are identical between human TTP and the ZFP36L2 (TIS11D) protein used in the original structure. The other colors represent progressively greater amino acid differences between the two proteins, ranging from minimally different (aquamarine, upper right), through green, yellow, and orange, with orange representing the most marked amino acid differences.

to be a fairly close approximation of the final TTP TZF domain structure in complex with its RNA target.

One of the striking aspects of this model is the residues within the TTP TZF domain peptide that are identical to those in the ZFP36L2 peptide, which are shown in dark blue in Fig. 4. It is apparent that essentially all of the amino acid residues that are in direct contact with the RNA are identical to those in the ZFP36L2 peptide, whereas those residues that are progressively less well conserved (ranging in order of conservation from aquamarine [best conserved] through green, yellow, and orange [least conserved]) are far removed from the RNA contact surfaces. This model suggests that the RNA contact domain of the TTP peptide is likely to be identical in structure to that of ZFP36L2 (TIS11D), and that the differing residues on other faces of the peptide might be involved in potential specificity determinants, such as protein-protein interactions. The one potentially interfering interaction in this model is between the 5'-most U residue and the green amino acid in the upper left corner; however, from the data of Hudson and colleagues [32], this initial U is unstructured in the complex and thus can

assume other conformations than that pictured, to prevent interfering interactions with the peptide.

Other aspects of this model fit some of our experimental observations. For example, it is possible to mutate the middle A (U5) to C while retaining some TTP binding and deadenylating abilities (WSL and PJB, unpublished data). This fits with the apparent position of U5 near a 'hole' in the middle of the structure shown in Fig. 4, which represents part of the inter-finger linker region. Conversely, mutations of either of the two A residues in this sequence (A3 and A7) were not well tolerated, and they are critical in the structure to stacking interactions with hydrophobic residues in the peptide. Mutations within these critical hydrophobic amino acids were also not well tolerated in terms of RNA binding and promoting mRNA deadenylation [35]. It will be interesting in the coming years to extend these analyses to other residues in the TZF domain, particularly in the highly conserved inter-finger linker region, which contains two basic residues thought to be critical for the nuclear import of the peptide [36].

Given that these studies have all agreed that the central optimal binding motif is 5'-UUAUUUAUU-3', it is of interest to compare this sequence with the naturally occurring physiological targets of TTP found in the TNF- α and GM-CSF mRNAs. Figure 5 shows the central ARE sequences from all known mammalian orthologs of the TNF- α and GM-CSF mRNAs [37]. It should be noted that the extreme conservation of these motifs falls apart in both the 5' and 3' direction in both TNF- α and GM-CSF mRNAs. By far the most popular mammalian pattern in the TNF- α mRNA is exemplified by the human sequence and those of nine other mammals, in which there are five overlapping versions of the nonamer-binding site. Within the five overlapping nonamers are several non-overlapping patterns. It will be of great interest to determine how many intact TZF peptide and intact TTP protein molecules can bind to this sequence, because it might be expected that the overlapping nonamers might prevent binding to some of the neighboring nonamers by steric hindrance. It is also of interest that several other mammals have different patterns and total numbers of these nonamers (Fig. 5a); again, it will be of interest to determine whether there are species differences in the number of TTP molecules that can occupy these domains, perhaps resulting in species differences in TTP effects on mRNA turnover rates.

The situation is somewhat different with the GM-CSF transcript, because the common mammalian pattern is to have three densely overlapping nonamers (Fig. 5b). The pig is an outlier in this case, with three more widely spaced nonamers. However, GM-CSF also has a completely conserved 5'-UAUUUAU-3' heptamer, with miscellaneous bases in the 1 and 9 positions in the

transcription-factor-binding sites within the promoter and single intron that are involved in this rapid and transient transcriptional response, but the binding proteins remain to be identified [40].

In contrast to this rapid but transient induction of the TTP mRNA in response to various stimuli, the protein seems to be surprisingly stable. In a recent study from our laboratory in which a TTP antiserum was used to probe western blots from mouse RAW264.7 macrophages after stimulation with LPS [41], the protein was almost undetectable in normally growing, unstimulated cells. This was compatible with parallel studies in normal mouse tissues, in which extraordinary amounts of tissue protein were needed in western blots to be able to detect immunoreactive protein. However, within 30–60 min of stimulation with LPS, readily detectable protein began appearing in the cytosol, reaching a peak value after about 2–4 hours. Surprisingly, despite the relative lability of the mRNA in most cell types, the protein remained very stable in the cells for many hours thereafter; it was still much higher than baseline levels after 6 hours, and was still readily detectable after 24 hours. Therefore, *de novo* biosynthesis of protein is clearly a major mode of TTP regulation, with manyfold increases occurring within a few hours after stimulation with LPS. Nonetheless, the reversal of this process seems to be rather slow.

Another interesting aspect of the protein accumulation experiments is that, as the protein was being synthesized and accumulated in the cellular cytoplasm, its apparent molecular mass on SDS gels continued to increase in an incremental manner, compatible with a stoichiometric increase in its phosphorylation. This occurred more slowly than the generally accepted model of protein phosphorylation, in which a previously synthesized protein is acted upon within a few minutes by a newly activated protein kinase. Possible mechanisms for this slow but profound increase in phosphorylation include the following: first, the protein was initially in the wrong cellular compartment to serve as a kinase substrate, and only became accessible after a lag of 1–2 hours; second, the protein was protected from phosphorylation by binding proteins or even intramolecular folding events; third, the protein was phosphorylated by one or more protein kinases that were either slowly activated by LPS or whose biosynthesis was also stimulated by LPS; fourth, the phosphorylation of the protein was increased by the activity of constitutively active cellular protein kinases in the setting of phosphatases that were either inactivated or destroyed in response to stimulation with LPS; or fifth, some combination of these events.

Elucidation of these mechanisms is of increasing interest because the effect of protein phosphorylation on TTP behavior has begun to be elucidated in a flurry of recent

studies. In our initial report on TTP phosphorylation [5], we demonstrated that both major bands of phosphorylated TTP expressed in intact fibroblasts, with or without stimulation with fetal calf serum, contained only phosphoserine. We identified a single major serine phosphorylation site in that study (S220 in the mouse, corresponding to S228 in the human protein) that was a substrate for MAP kinases both in intact cells and in cell-free systems. In both cases – that is, in intact cells and in cell-free assays – this phosphorylation event changed the migration behavior of the protein in SDS gels, compatible with a stoichiometric increase in phosphorylation. However, because nothing was known about TTP's physiological function at that point, we were unable to conclude anything about the effect of this phosphorylation event on protein behavior except to say that the absence of this phosphorylation site did not seem to affect TTP's ability to translocate from the nucleus to the cytosol in response to stimuli such as serum.

Several more recent studies have added to our knowledge of TTP phosphorylation and its effects on protein behavior. In one study we found that TTP could serve as a substrate for the p38 protein kinase and that global dephosphorylation of TTP in a cell-free system with alkaline phosphatase seemed to increase its binding affinity for an ARE-containing RNA substrate [42]. Clark and colleagues [43] demonstrated that TTP could serve as a substrate for the MAP kinase-activated protein kinase 2 (MK2), although these authors did not detect direct phosphorylation of TTP by the p38 kinase. Johnson and colleagues [44] demonstrated that TTP could bind to the cellular protein 14-3-3, and that this binding could be influenced by the phosphorylation status of the protein, specifically at serine 178 in the mouse protein (corresponding to serine 186 of the human protein). They also demonstrated that the binding to 14-3-3 protein promoted the localization of TTP to the cellular cytoplasm. Very recently, Chrestensen and colleagues [45] identified the same serine 178 as a major phosphorylation site for MK2; they also identified serine 52 as another major site and showed that these two phosphorylations created functional binding sites for 14-3-3. Finally, Stoecklin and colleagues [46] showed that phosphorylation of TTP by MK2 on serines 52 and 178 led to 14-3-3 binding, which in turn led to the exclusion of TTP from arsenite-induced stress granules. Although the 14-3-3 interaction still permitted binding of TTP to RNA, it inhibited the TTP-dependent degradation of ARE-containing RNA substrates.

Taken together, these data suggest a model in which TTP activity against ARE-containing mRNAs can be modulated by the activation of p38 and the MK2-induced phosphorylation of TTP, leading in turn to 14-3-3 association and cytoplasmic sequestration as well as the inhibition of mRNA degradation. The relevance of direct

p38 phosphorylation is unclear, although some of the minor sites identified by Christensen and colleagues [45] might have been sites for the p38 kinase. It will be interesting to determine how this model works in intact organisms; this should now be capable of investigation, given the availability of MK2-deficient mice [47]. In fact, as pointed out by Mahtani and colleagues [43], mouse spleen cells from MK2-deficient mice seemed to express normal levels of TNF- α mRNA, which exhibited apparently normal stability after LPS stimulation [47].

The studies indicating association of TTP with 14-3-3 protein highlight another possible mode of regulation of TTP activity, namely association and dissociation from binding proteins. In addition to 14-3-3, previous studies have identified interactions of TTP with TIA-1 [48] and the nucleoporin CAN/Nup214 [49]; in the latter study, the interaction again seemed to regulate the intracellular localization of TTP. Functional studies have suggested interactions with the nuclear export protein CRM1 [50], although direct binding interactions were not demonstrated in that study. Chen and colleagues [51] demonstrated an association of TTP with components of the exosome, suggesting a role for this structure in the degradation of deadenylated ARE-containing mRNAs. Finally, a recent two-hybrid analysis by our group (Blackshear PJ, unpublished data) identified a large number of previously unidentified potential interacting proteins, each of which needs to be painstakingly validated by other interaction methods. We expect to see the identification of many such interacting proteins in the immediate future, and many of these binding events will undoubtedly have effects on the cellular physiology of the protein.

Polymorphisms in the human TTP gene (*ZFP36*)

It is possible that severe mutations in TTP coding sequences could prevent or decrease the expression of mature transcripts, interfere with splicing of the single intron, or lead to frame-shift or stop-codon mutations. These could have major effects on TTP's ability to bind to TNF- α transcripts and destabilize them. Linkage studies with less severe TTP polymorphisms could provide insight to the treatment and/or diagnosis of human disorders associated with excess TNF- α , such as rheumatoid arthritis or Crohn's disease. Although such linkage studies are yet to be completed, the first step in performing them is to identify polymorphisms. We have taken this first step and identified several single-nucleotide polymorphisms (SNPs) in the human TTP gene, *ZFP36* [52].

As part of the NIEHS Environmental Genome Project (EGP), *ZFP36* was resequenced in the genomic DNA from 92 anonymous subjects (Coriell collection and Coriell Polymorphism Discovery collection; see reference [52] for further details). Resequencing identified 10

polymorphisms and expressed sequence tag (EST) searches identified an additional four potential SNPs in *ZFP36*. These are summarized in Table 1, and a schematic depiction of the SNP positions on the human gene is shown in Fig. 6.

Polymorphisms in the promoter region and single intron of TTP are of particular interest because both of these regions are necessary for the proper regulation of expression [8,9]. Four polymorphisms were identified in the promoter region. The polymorphism *ZFP36*2* at base 359 in the promoter was the most common SNP identified; it was present at 47% in the EGP subjects. The other three promoter SNPs were *ZFP36*1* (found in 1.8% of the EGP subjects), *ZFP36*3* (3.1%), and *ZFP36*4* (0.6%). Two intronic polymorphisms were also found in this group. *ZFP36*5* and *ZFP36*6* were present in the 92 EGP subjects at frequencies of 0.5% each.

Six polymorphisms were identified in the protein-coding domains by resequencing; all were in exon 2. Three of the SNPs were identified by resequencing the EGP subject DNA. These SNPs were present at frequencies of 0.6% for *ZFP36*7*, 6.2% for *ZFP36*8*, and 4.2% for *ZFP36*9*. Of these three SNPs, only *ZFP36*7* resulted in an amino acid change from proline to serine. Because this represented a non-conservative change, the frequency of *ZFP36*7* was determined in 422 North Carolina subjects of varying ethnicities; it was found in 2.4% of this population [52].

Three other potential protein-coding-domain polymorphisms were identified by EST searches. One of the SNPs, *ZFP36*11*, was found in 4.2% of the ESTs examined. This SNP resulted in a non-conservative amino acid change from glycine to cysteine. Although this SNP is within the TZF RNA-binding domain (in the 18 amino acid 'linker' between the two zinc fingers), it had no detectable effect on the RNA-binding ability of TTP in a cell-free gel-shift assay. The other two SNPs, *ZFP36*12* (present in 4.1% of ESTs examined) and *ZFP36*13* (11%), did not produce an amino acid change.

An ARE in the 3' untranslated region (UTR) of TTP confers instability on the mRNA, as determined by deletion studies (WSL and PJB, unpublished data). This region was also examined for polymorphisms. Two polymorphisms, *ZFP36*10* and *ZFP36*15*, were identified within the 3' UTR. *ZFP36*10* was identified by resequencing the EGP subject DNA. This polymorphism was the second most frequently occurring polymorphism (frequency 7.6% in EGP subjects). *ZFP36*10* lies within a region of the transcript that is highly conserved among mammals and therefore might be significant in terms of altering TTP mRNA stability. The other 3' UTR polymorphism, *ZFP36*15*, was identified only by EST searches (present

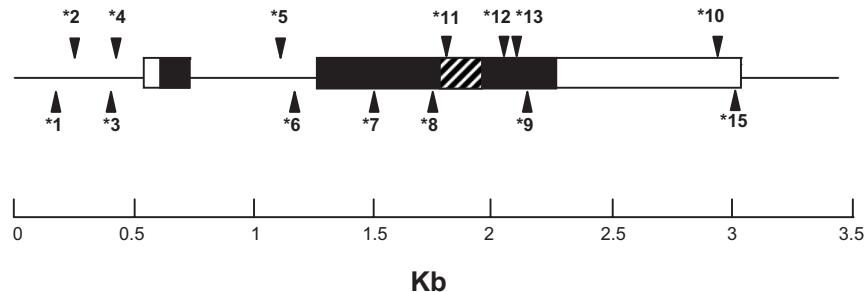
Table 1

Polymorphisms in ZFP36 encoding human tristetraprolin

Polymorphism	Location	Base	Change	Sequence	Amino acid change	Variant allele frequency in EGP subjects (%) (n = 92)	Variant allele frequency in ESTs (%)
ZFP36*1	Promoter	316	C→A	CCCCC(C/A)ATCCG		1.8	
ZFP36*2	Promoter	359	A→G	CGGTC(A/G)CGGCT		47	
ZFP36*3	Promoter	490	C→A	CCGGC(C/A)CCGGC		3.1	
ZFP36*4	Promoter	492	C→T	GGCCC(C/T)GGCCC		0.6	
ZFP36*5	Intron	1226	G→A	GGGAA(G/A)CCGGG		0.5	
ZFP36*6	Intron	1256	C→G	TAAGG(C/G)CTCGG		0.5	
ZFP36*7	PCD (ex.2)	1525	C→T	CGGGA(C/T)CCTGG	P37→S	0.6 ^a	0/127
ZFP36*8	PCD (ex.2)	1725	C→T	TCGCG(C/T)TACAA	R103→R	6.2	2/127 (1.6%)
ZFP36*9	PCD (ex.2)	2235	T→C	CCCTC(T/C)GTACA	S273→S	4.2	1/69 (1.4%)
ZFP36*10	3' UTR	2980	Del TT (7T→5T)	TTTTT(delTT)GTAAT		7.6	62/249 (26%)
ZFP36*11	PCD (ex.2)	1807	G→T	GCCTG(G/T)GCGAG	G131→C		5/118 (4.2%)
ZFP36*12	PCD (ex.2)	2112	C→T	GCCTT(C/T)TCTGC	F232→F		4/97 (4.1%)
ZFP36*13	PCD (ex.2)	2184	C→A	AGGGC(C/A)ACTCC	A256→A		10/94 (11%)
ZFP36*15	PCD (ex.2)	3059	A→C	TGCCT(C/T)CCGCT			17/232 (7.3%)

The base numbers correspond to the following GenBank accession numbers: ZFP36 gene, M92844; tristetraprolin cDNA, NM_003407.1; tristetraprolin protein, NP_003398.1. Polymorphism numbering is consistent with that in [52]. Base refers to the base number in the genomic sequence M92844. The polymorphic changes are indicated as follows: A (original base or amino acid)→G (polymorphic base or amino acid), or (A/G) in the sequence column. EGP, Environmental Genome Project; ex. 2, exon 2; PCD, protein coding domain; 3'-UTR, 3'-untranslated region. Table modified from [52]. ^aThis SNP was also found in 2.4% of subjects from Durham, North Carolina (see the text). The change labeled 'Del TT' refers to the removal of two T residues, changing the normal seven consecutive T residues to five in the variant sequence.

Figure 6



Schematic representation of the human tristetraprolin (TTP) gene (ZFP36) and its polymorphisms. The two exons of ZFP36 are shown as boxes, whereas the flanking regions and intron are indicated by a thin line. Open boxes represent untranslated regions, solid filled boxes represent protein-coding regions, and the hatched region represents the tandem zinc finger domain. The positions of the polymorphisms listed in Table 1 are indicated by arrowheads. Kb, kilobases. The data are modified from [52].

in 7.3% of the ESTs examined). Both of these SNPs are potentially interesting in that they lie with a region of the TTP mRNA 3' UTR that probably contributes to the stability of this message.

Since the completion of the initial resequencing study, we have embarked on a more extensive study in subjects with various potentially related diseases. These include subjects with TNF-associated periodic inflammatory

syndrome (TRAPS); bronchial hyper-responsiveness to inhaled endotoxin; rheumatoid arthritis, both responsive and resistant to anti-TNF- α therapies; various subgroups of juvenile rheumatoid arthritis; psoriatic arthritis; and multiple sclerosis. If these subjects are added to the original 92 members of the EGP participants, then we will have resequenced the gene from a total of 507 subjects. Although analysis of these samples is not yet complete, we have identified an additional 20 potential SNPs, making the total number discovered so far about 35. Current efforts include assembling the known SNPs into haplotypes for association studies, determining SNP and haplotype frequency in a large number of normal subjects, and a biochemical examination of the SNPs and haplotypes for effects on the biosynthesis, activity and stability of the TTP protein itself.

It is of interest in this regard that long-term follow-up of the TTP hemizygous mice has shown that several mice older than 1 year have developed the full-blown TTP deficiency phenotype, apparently in a stochastic and unpredictable manner. This suggests that TTP hemizygosity in humans might be compatible with a completely normal life, but that in some cases the relative TTP insufficiency might lead to disease, perhaps in response to some environmental perturbation. We expect that a full-blown, autosomal recessive TTP deficiency syndrome would be severe and fatal in childhood in humans, but so far there are no known instances of such a condition in humans.

TTP and related CCCH proteins in humans

As noted above, there are now known to be three members of the TZF protein family in humans, and extensive blasting of the human genome and EST collections has not yielded any further members, despite the presence of a group of sequences of closely related fourth members in fish and frogs [53]. Much less is known about the physiological roles of these proteins in mammalian systems. As shown in Fig. 7a, all three members of the family can bind readily to a TNF- α ARE probe, as demonstrated by RNA gel-shift analysis. In addition, all three family members can promote the deadenylation of ARE-containing polyadenylated RNA probes, both in intact cell transfection systems and in cell-free deadenylation assays (Fig. 7b) [23,54]. This occurs whether or not the proteins are used to 'effectively activate' endogenous deadenylating activities in HEK-293 cell extracts, or co-transfected PARN in the same cells (Fig. 7b). This and other types of evidence suggest that all three proteins have similar roles to TTP in the physiology of some cell types; that is, they are capable of binding to specific ARE sequences in certain transcripts and promoting their deadenylation and degradation. Many questions remain, including the following. First, in what cell types does each protein function as an mRNA destabilizing factor, and in what physiological or

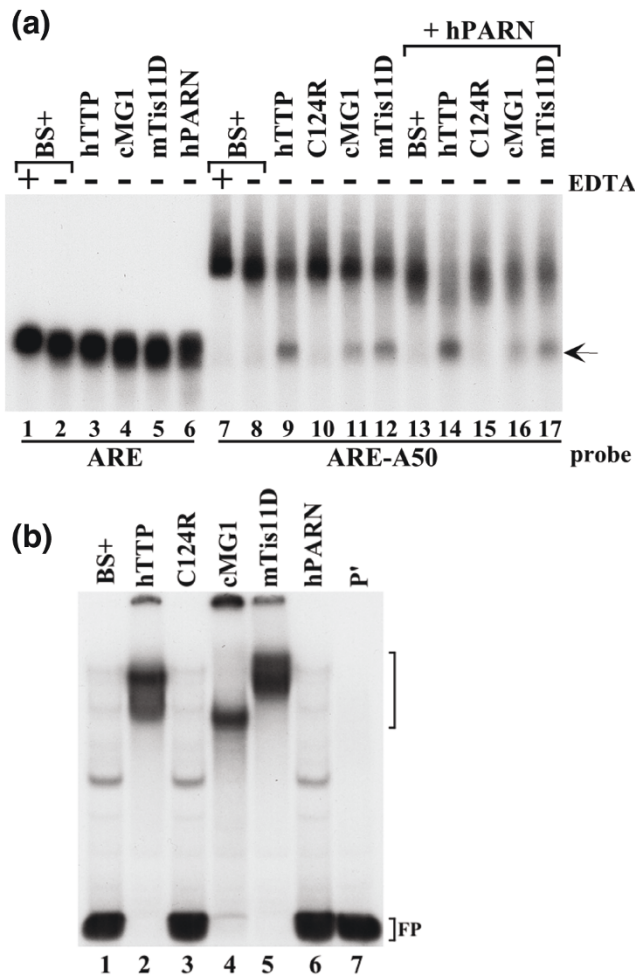
pathological situations? Second, how are these interactions regulated, by biosynthetic and post-translational events, as well as interactions with other cellular proteins? Third, what are the mRNA targets for each family member in normal physiology?

For *Zfp36L1* in mouse, we have recently shown that its complete deficiency leads to universal intrauterine death of the KO embryos, usually at about embryonic day 9–11 [55]. In most cases there was failure of chorioallantoic fusion, which undoubtedly leads to the death of the embryos. In the minority of cases in which fusion occurred, there seemed to be secondary failure of the placenta, leading to poor perfusion of the embryo, runting, widespread apoptosis, neural tube defects, and death. The presumed stabilized transcripts that lead to these abnormalities are not known, but they are the subject of continuing study in the laboratory. This embryonic lethal phenotype means, however, that elucidation of the physiological function of the *Zfp36L1* protein in cells and tissues from adult mice will require conditional KO strategies.

For *Zfp36L2* in the mouse, our attempts to create a conventional KO mouse led to a mouse in which a transcript is produced that contains a significant portion of the single intron as well as the complete second exon, apparently driven by the endogenous promoter (despite the presence of the *neo* gene in between) [56]. The result is that the tissues and cells of this mouse produce a protein that lacks the amino-terminal 29 amino acids and is expressed at a variable fraction of endogenous protein levels in different cells and tissues. Despite this unsatisfactory situation, a mouse was produced that has a very specific phenotype: complete female infertility of the KO mice, apparently due to arrest of the embryo after the two-cell stage. This seems to be a maternal effect, because KO embryos from heterozygous mothers seem to develop entirely normally. This is an unusual phenotype and seems to implicate *Zfp36L2*, and particularly its amino terminus, in maternal aspects of the earliest stages of embryonic development. The development of more conventional and complete KO mice is currently under way.

As part of our evaluation of *ZFP36* polymorphisms in humans, we have evaluated the transcript levels for all three family members in one human cell type, purified and cultured monocytes prepared from normal subjects and subjected to stimulation with LPS. It is difficult to compare expression levels of different proteins or transcripts by immunological and northern blotting procedures because of differences in antibody or probe affinity and for other reasons. However, with the use of real-time polymerase chain reaction (PCR) it is possible to make quantitative comparisons between different transcripts by using primer and probe sets that are

Figure 7



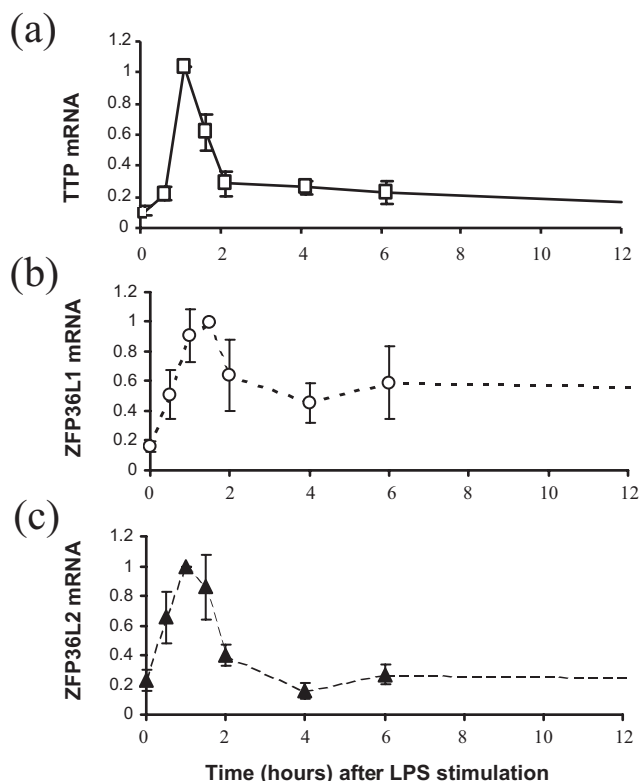
Effects of tristetraprolin (TTP)-related tandem CCCH zinc finger (TZF) proteins to bind AU-rich element (ARE)-containing probes and to promote their deadenylation. HEK-293 cells were maintained, and transient transfection of 1.2×10^6 cells with expression plasmid constructs in calcium phosphate precipitates was performed, as described [22]. To each plate of HEK-293 cells was added 0.2 μ g of the TZF protein expression constructs CMV.hTTP.tag (hTTP), a human TTP (hTTP) zinc finger mutant (C124R), CMV.cMG1.tag (cMG1), CMV.mTis11D.tag (mTis11D), 0.1 μ g of human poly(A) exonuclease (hPARN) expression plasmid CMV.hPARN.flag (hPARN), or plasmid DNA alone (BS+). The zinc finger protein expression constructs were transfected either with vector alone or together with CMV.hPARN.flag; vector DNA (BS+) was added to each transfection to make the total amount of co-transfected DNA 5 μ g per plate. Cytosolic extracts were prepared and used in deadenylation assays as described [23]. (a) Extracts (10 μ g of protein per sample) were incubated with probes ARE or ARE-A50 at 37°C for 60 min in the presence (+) or absence (-) of 20 mM EDTA, as indicated. The samples were processed as described previously [23]. The arrow indicates the migration position of the ARE probe (lanes 1-6) and the deadenylated product of probe ARE-A50 (lanes 9, 11, 12, 14, 16 and 17). (b) The extracts used in lanes 7-13 of (a) were incubated with the ARE-A50 probe and used in a gel-shift assay. Lane 7 (P') was loaded with probe alone (digested with RNase T1). The migration positions of the zinc finger protein-RNA complexes are indicated by the bracket to the right of the gel, and the position of the free probe (FP) is also indicated. The bands present in the gel in lane 1 represent endogenous HEK-293 cell proteins shifting the probe; note that this pattern is identical in lane 3, representing a zinc finger mutant of TTP, and in lane 6, representing hPARN alone.

carefully matched for PCR amplification efficiencies and fluorescence intensities. Using this technique, we have compared the expression profiles and levels of transcripts encoding TTP, ZFP36L1, and ZFP36L2 in normal human monocytes stimulated with LPS, to determine the approximate percentages of expression of each transcript in the control and stimulated states.

Purified human monocytes (from Dr Keith Hull and Dr Dan Kastner, National Institutes of Health) were treated for various times up to 24 hours with LPS (1 ng/ μ l) or phosphate-buffered saline (PBS) as a control. The cells were harvested, and the RNA was extracted, treated with DNase, and reverse transcribed to cDNA; then 5 μ l of the cDNA was subjected to Taqman analysis (Applied Biosystems 7900 instrument) using primers and fluorescently-labeled probes (Applied Biosystems Assays On Demand) specific for each of the genes of interest. The primer/probe sets were previously validated to detect

only the gene of interest and to have similar PCR amplification efficiencies and fluorescence intensities, as determined by experiments showing that equivalent C_t values were obtained with the same cDNA copy number for each gene and primer/probe set; this allowed comparisons to be made of the relative expression levels of each of the transcripts.

For the data shown in Fig. 8, RNA from LPS-stimulated or PBS-stimulated monocytes from five healthy human subjects was analyzed for expression of TTP, ZFP36L1, and ZFP36L2 transcript levels, along with four internal control transcripts (18S rRNA, PSMB6, HNRPL, and PSMD7). The $\Delta\Delta C_t$ method of analysis [57] was used to determine changes in gene expression. The method of normalizing the gene expression data is based on research in [58], in which the geometric mean of several internal controls was used to normalize the gene expression data. The internal controls in the present study

Figure 8

Tristetraprolin (TTP), ZFP36L1, and ZFP36L2 expression patterns in human monocytes stimulated with lipopolysaccharide (LPS). Purified monocytes from healthy human subjects ($n = 5$) were stimulated with LPS (or phosphate-buffered saline as control). Total cellular RNA from the monocytes was converted to cDNA and analyzed by real-time polymerase chain reaction for (a) TTP, (b) ZFP36L1, and (c) ZFP36L2 expression levels. Resulting C_t values were normalized to the geometric mean of four internal control transcripts and then to corresponding samples from PBS-treated cultures at the same time points, then converted to $2^{-\Delta\Delta C_t}$. The normalized values were then expressed as a fraction of the mean value at which maximum expression occurred ($t = 1$ hour for TTP and ZFP36L2; $t = 1.5$ hours for ZFP36L1). These were then expressed as means \pm s.e.m.

were selected on the basis of the previous identification of these genes as being stably expressed in adult and fetal tissue [59] and the absence of evidence from previous work that these genes were affected by LPS. Furthermore, to minimize plate-to-plate variability between real-time PCR assays, the signal from LPS-treated RNA was normalized to the signal from PBS-treated RNA from the same subject (at the same time points), assayed together on the same plate.

Initially, all data were expressed as a fraction of maximal expression, set at 1.0. Stimulation of human monocytes with LPS caused a rapid increase in the accumulation of TTP mRNA, reaching about 16-fold that in the control by 60 min, then rapidly declined again (Fig. 8a). This is similar

to the profile seen in mouse macrophages in response to LPS [17].

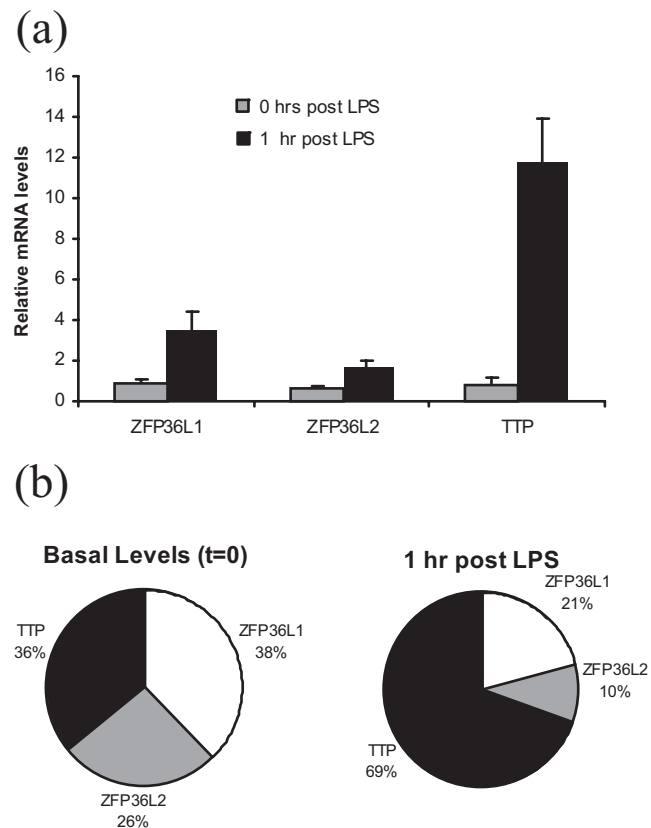
Analysis of ZFP36L1 transcript expression patterns in the monocytes after stimulation with LPS showed a similar pattern to that of TTP mRNA, but the peak value was not reached until 90 min, and the return to baseline was slightly slower (Fig. 8b). In addition, ZFP36L1 transcripts did not decrease to basal levels, even after 24 hours. Although the temporal sequence of transcript accumulation was similar to that of TTP, the maximal increase at 90 min was only about 4.4-fold the control, unstimulated values, compared with the 16-fold increase with TTP.

Analysis of ZFP36L2 mRNA levels after LPS stimulation in the same samples exhibited an expression profile similar to that of TTP mRNA (Fig. 8c). Again, the overall fold increase in expression was much less than that of TTP, with transcript levels increasing to less than threefold that of the control at the maximal time point (Fig. 8c).

Although the temporal expression patterns of each of the TTP-related transcripts were similar after stimulation with LPS, the mRNA levels relative to one another were different (Fig. 9). At baseline ($t = 0$), all three transcripts were present at similar levels, each accounting for about one-third of the total 'TTP transcript equivalents' in unstimulated human monocytes (Fig. 9). However, 1 hour after LPS stimulation, TTP mRNA levels increased to about threefold those of ZFP36L1 transcripts, and to about sevenfold those of ZFP36L2 transcripts. Thus, after stimulation for 1 hour with LPS in normal human monocytes, TTP accounted for about 69% of the total TTP equivalents, ZFP36L1 21%, and ZFP36L2 10% of the total TTP-related transcripts.

These data have important implications for potential treatments directed at TTP specifically as an approach to anti-TNF- α treatment. In resting, unstimulated monocytes, each of the family members may contribute approximately equally to the turnover of the ARE-containing target transcripts, whereas the TTP effect may become predominant after stimulation of the innate immune system. Interfering with TTP itself, for example by completely inhibiting its biosynthesis, might have less effect than expected because of partial compensation by other family members expressed in the same cell. From the TTP KO mouse experiments, there was no apparent compensatory increase in the expression of the other two family members in cells and tissues from the KO mice [11]. Nonetheless, their presence at approximately equal molar concentrations suggests that they might well contribute to normal rates of mRNA deadenylation and stability in physiological circumstances. Many other factors could modify this conclusion, including major

Figure 9



Relative basal and stimulated levels of tristetraprolin (TTP), ZFP36L1, and ZFP36L2 transcripts in cultured human monocytes. The real-time polymerase chain reaction (PCR) values were normalized to the geometric mean of four internal transcript controls and against PBS-treated cells at corresponding time points, then converted to $2^{-\Delta\Delta Ct}$ to obtain expression level values for the TTP, ZFP36L1, and ZFP36L2 transcripts, shown in (a) as means \pm s.e.m. from cultures derived from five individual subjects. These expression levels from the three transcripts can be compared with each other within subjects, because the primer/probe sets for each gene yielded equivalent PCR amplification efficiencies and fluorescence intensities, and the data were normalized to control for plate-to-plate variations. The basal levels are similar for each of the three genes in resting monocytes, shown as 0 hours after lipopolysaccharide (LPS) in (a) and $t = 0$ in (b). LPS treatment for 1 hour stimulated the expression of all three genes; however, after 1 hour of exposure to LPS, TTP levels were increased most markedly (a and b, right panel). In unstimulated cells, mean levels of all three transcripts representing TTP 'equivalents' were approximately equal (b, left panel). However, after 1 hour of stimulation with LPS, TTP represented about 69% of TTP equivalents, compared with 21% for ZFP36L1 and 10% for ZFP36L2.

differences in the translation of the transcripts, differences in subcellular localization, differences in post-translational modification, and differences in associated binding proteins.

Nonetheless, these findings might help to explain why the phenotype of the Δ ARE mice is so much more severe than

that of the TTP KO mice [13]. In the homozygous Δ ARE mice there would be no target ARE in the TNF- α transcript for any of the three family members to bind to, whereas in the TTP KO mice the other two family members could bind to the TNF- α ARE and decrease its stability. As the other family members are knocked out, presumably by using conditional KO strategies, it may be possible to isolate macrophages deficient in one, two, and three family members to determine the potential additive effects of each member on TNF- α transcript stability. It will also be important to explore the expression of the various family members in diseased tissues of humans and mice, to determine whether certain cell types in, for example, the inflamed joints of rheumatoid arthritis might be overexpressing local TNF- α and other inflammatory cytokines because of a relative local insufficiency of TTP or its family members.

Conclusions

Although more than 6 years elapsed between the cloning of the cDNA for TTP and the discovery of its role in regulating TNF- α expression, the years since that connection was made have yielded many insights into the functions of this fascinating family of proteins. This work by many groups has culminated most recently in the striking structure of the TZF domain in complex with the nine-base ARE target [32]. Can this interaction represent a novel target for anti-TNF- α therapies? It has been difficult to pursue studies of this type so far, because one would ideally be searching for small molecules that would mimic or potentiate the binding of TTP to its TNF- α ARE target, resulting in mRNA destabilization and decreased TNF- α secretion. Such molecular targets might also be difficult to use therapeutically, because agents that acted like TTP, or increased TTP's ability to destabilize TNF- α mRNA, might be expected to have similar effects on the GM-CSF mRNA in other cell types, perhaps with deleterious effects on GM-CSF functions. Conversely, inhibitors of this interaction might be expected to increase GM-CSF secretion, perhaps a beneficial response in neutropenic states, but at the same time have the potentially harmful side effect of inhibiting TNF- α mRNA degradation. Nonetheless, we view these approaches as potentially useful, particularly with the development of convenient, low-volume fluorescence assays for the binding of the TZF domain to RNA substrates [31,38]; these could conveniently be adapted to high-throughput formats.

An alternative approach would be to try to identify small molecules that specifically penetrate macrophages and monocytes and stimulate TTP biosynthesis. It is clear that transcription of the TTP gene *ZFP36* is regulated differently from that of the other two family members, and it might be possible to uncover compounds that stimulate its biosynthesis, specifically in macrophages, that would

not affect either TNF- α biosynthesis or that of the other two family members. Such a molecule might be a useful prototype or lead compound for a novel approach to anti-TNF- α therapies, which have proven to be so useful in the treatment of rheumatoid arthritis and Crohn's disease in the past several years [60].

Competing interests

The author(s) declare that they have no competing interests.

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