Interactions of CCCH zinc-finger proteins with mRNA.

Non-binding Tristetraprolin mutants exert an inhibitory effect on degradation of
AU-rich element containing mRNAs.

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Summary

Tristetraprolin (TTP), the prototype of a small family of CCCH tandem zinc finger (TZF) proteins, is a physiological stimulator of instability of the mRNAs encoding tumor necrosis factor α (TNFα) and granulocyte-macrophage colony stimulating factor in certain cell types. TTP stimulates mRNA turnover after binding to class II AU-rich elements (ARE) within the 3′-untranslated region of both mRNAs. In turn, this binding is dependent upon the key CCCH residues in the TZF domain. To evaluate other primary sequence requirements for ARE binding in this novel mRNA binding domain, we mutated many of the conserved residues within the TZF domain of human TTP, and evaluated the effects of these mutations on RNA binding in a cell-free system and TTP-induced mRNA instability in cell transfection experiments. These mutations revealed a number of conserved amino acids that were required for binding, and begin to define the primary protein sequence requirements for this novel mRNA binding motif. Unexpectedly, all of the point mutations that prevented TTP binding to RNA also caused an increase in steady-state levels of ARE-containing mRNAs in cell transfection experiments. Actinomycin D experiments suggested that this effect was due to inhibition of mRNA turnover. Although expression of the mutant form of TTP could also inhibit the destruction of TNFα mRNA by wild-type TTP, the primary mechanism did not involve heterodimerization with wild-type TTP, since the 293 cells used in these studies express no detectable endogenous TTP. These data suggest that TTP may act, at least in part, by physically interacting with an enzyme activity or protein complex and functionally stimulating its ability to deadenylate class II ARE-containing mRNAs.
The CCCH zinc finger motif has been found in proteins from organisms ranging from man to yeast (1-11). A small subset of these proteins contains two CCCH zinc fingers with the following characteristics: The fingers are 18 amino acids apart; they have the internal spacing Cx₈Cx₅Cx₃H, where x indicates variable amino acids; and both fingers have a highly conserved lead-in sequence, R(K)YKTEL. Three such proteins have been identified to date in mammals, with a fourth member recently discovered in *Xenopus* and fish (2,12,13). We will refer to this small group of proteins as the tandem zinc finger (TZF₁) domain CCCH proteins.

Our group has begun to elucidate a function for this group of proteins by analyzing the phenotype of mice deficient in tristraprolin (TTP), the prototype of this group of proteins. These animals develop a complex phenotype consisting of cachexia, dermatitis, conjunctivitis, destructive arthritis, myeloid hyperplasia, and autoimmunity (14). Virtually all aspects of this phenotype could be prevented by the repeated injection of antibodies to tumor necrosis factor α (TNFα), implicating an effective excess of circulating TNFα in the pathogenesis of this condition (14). This was confirmed by studies showing that the phenotype could be largely prevented by interbreeding the TTP knockout mice with mice lacking both types of TNFα receptors (15,16). Macrophages derived from the knockout mice elaborated more TNFα into the culture medium than did macrophages from control animals, and this increased secretion was accompanied by increased accumulation of TNFα mRNA in the cells (17). TNFα mRNA was markedly stabilized in the cells from the TTP-deficient animals (18). These findings implicated TTP as a stimulator of TNFα mRNA turnover in normal macrophage physiology. More recently, we showed in similar experiments in bone marrow-derived stromal cells that TTP exerts a similar, physiological effect on the stability of the mRNA encoding a second cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) (15). Over-
expression of this growth factor may contribute to the myeloid hyperplasia characteristic of the TTP knockout phenotype (16).

The mechanism of TTP's effect on TNFα and GM-CSF mRNA stability is not understood. TTP can bind directly to the so-called class II AU-rich elements (ARE) in the TNFα and GM-CSF mRNA 3'-untranslated regions (3 UTR) (18). This binding is dependent on the integrity of each of the two zinc fingers, in that mutation of a single cysteine to arginine in either zinc finger completely abrogated binding of TTP to these ARE probes (19). In transfection experiments in 293 cells, TTP caused the accelerated breakdown of TNFα and GM-CSF mRNAs, and non-binding mutants failed to exert this effect (19). These data indicate that the first and possibly rate-limiting step in TTP action on class II ARE containing mRNAs is direct binding to the ARE, followed by stimulation of deadenylation and subsequent further destruction of the mRNA body.

We have begun to explore the structure-function relationships between the primary sequence of the TZF domain and mRNA binding. Apparently normal binding still occurred when the TTP protein was truncated to a 77 amino acid fragment that contained the TZF domain and seven flanking residues on either side (20). By investigating the binding of RNA to TZF domains from various CCCH proteins, we began to characterize the extent of natural variation that could be tolerated and still maintain RNA binding activity (20). In the present studies, we have extended these findings by generating and testing a series of site-directed mutants of the TTP TZF domain. Unexpectedly, all of the non-binding mutants shared the ability to inhibit the initial step, presumably deadenylation, in the breakdown of class II ARE-containing mRNAs.

**Experimental procedures**
1. **Plasmid constructs.**

The expression plasmids CMV.mTNFα (127-1325), CMV.mTNFα (1-1627), CMV.GM-CSF and CMV.IL-3 have been described previously (19,23).

The TTP zinc finger mutants of expression plasmid CMV.hTTP.tag were made by the PCR primer-overlapping mutagenesis technique (21). Correct sequences of all intended mutations were confirmed by dRhodamine Terminator Cycle Sequencing (Perkin-Elmer, Foster City, CA). The numbering system for the TTP mutants used the GenBank RefSeq for TTP, NP_003398.

2. **Transfection of HEK 293 cells and northern analysis.**

HEK 293 cells (referred to as 293 cells) were maintained, and transient transfection of 1.2 x 10^6 cells with plasmid constructs in calcium-phosphate precipitates was performed, as described (20). In some experiments, pXGH5 (Nichols Institute Diagnostics, San Juan Capistrano, CA) was co-transfected to monitor transfection efficiency. Assays of released human growth hormone (HGH) were performed as described (22). In most experiments, the cells were co-transfected with various CCCH protein-expressing plasmids. Details of the construction of these plasmids have been described (19,20).

Twenty-four h after the removal of the transfection mixture, aliquots of cell culture medium were analyzed for HGH according to the manufacturer's protocol. Total cellular RNA was then harvested from the 293 cells using the RNeasy system (Qiagen, Valencia, CA). Northern blots were prepared as described (1). Blots were hybridized as indicated with a random-primed, α-^32^P-labeled cDNA probe of mouse TTP (1); a ~ 1 kb NarI-BglII fragment of a mTNFα cDNA (19); a 422 bp Sall-EcoRV 5' fragment of mouse GM-CSF cDNA (from plasmid CMV.mGM-CSF); and a 468 bp
SalI-XbaI 5' fragment of mouse IL-3 cDNA (from plasmid CMV.mIL3). Some blots were also hybridized with an $\alpha^{32}$-P-labeled GAPDH cDNA probe (17), or an $\alpha^{32}$-P-labeled cyclophilin cDNA probe (19) to monitor gel loading.


a. Preparation of RNA probes

Plasmid pTNF$\alpha$ 1309-1332 (bp 1309-1332 of GenBank accession number X02611) was constructed as described (19).

Plasmid pGM-CSF-ARE (bp 3399-3453 of GenBank accession number X03020), containing the AU-rich element in the mouse GM-CSF 3 UTR, 5'

AUUUAAUUUAUAUUUAUUUAUUUUUUAAAUUUUAUUUUAAUUUUAAUUUUAAUU

U-3, was constructed by inserting double-stranded oligonucleotides into the EcoRV-XbaI cloning sites of pSK-. Correct sequences of all plasmid inserts were confirmed by dRhodamine Terminator Cycle Sequencing (Perkin-Elmer).

To label RNA transcripts with [$\alpha^{32}$P]UTP (800 Ci/mmol), the above plasmids linearized with XbaI were used as templates, and the Promega Riboprobe in vitro Transcription Systems protocol was employed. The resulting products were separated from the free nucleotides through G50 columns.

b. Cross-linking of proteins to RNA
Cytosolic extracts were prepared from 293 cells transfected with plasmids expressing hTTP, or mutant zinc finger proteins, or vector. Cross-linking assays were performed using extracts (5 µg of protein) incubated with 1.5 x 10^6 cpm of RNA probe as described (19,20).

c. Western blotting.

Cell extracts (5 µg of protein) were mixed with 1/5 volume of 5X SDS sample buffer (19), boiled for 5 min, then loaded onto 12% SDS-PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in tris-buffered saline/0.3% Tween 20 (TBS/T) with a polyclonal antiserum HA.11 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:10,000).

d. RNA electrophoretic mobility shift assay.

Cytosolic extracts prepared from 293 cells transfected with either vector alone or expression constructs driven by the CMV promoter (5 µg of protein) were incubated with 2 x 10^5 cpm of RNA probe as described (19,20).

Results

1. Effect of mutations of conserved amino acids within the TZF domain on the ability of TTP to bind to ARE probes and to promote TNFα mRNA degradation.

In addition to the key amino acids CCCH in the tandem Cx₈Cx₅Cx₃H zinc finger motifs, there are many conserved amino acids within the TZF domains in members of the CCCH protein family (see Fig. 1). To begin to evaluate the importance of these conserved amino acids for the ability of TTP to promote ARE binding and class II ARE-containing mRNA degradation, we evaluated point mutants of these conserved amino acids in the CMV.hTTP.tag construct. We showed previously that a single C to R mutation in either the first (C124R) or the second (C147R) zinc finger of human
TTP (using the numbering system shown in Fig. 1) prevented binding of the mutant protein to the TNFα ARE (19). In the present study, we mutated the remaining CCCH residues in both fingers. Mutations in any one of these residues made TTP unable to bind to a TNFα mRNA ARE probe in a gel mobility shift assay (Fig. 2A, lanes 2-8), including mutants in which the third C in each finger was changed to H at positions 124 or 162 of hTTP (Fig. 2A, lanes 3 and 7). The lack of RNA binding seen with this collection of mutants was also demonstrated using a probe representing the other known physiological target of TTP, the GM-CSF mRNA ARE (Fig. 2B). The roughly equivalent expression of proteins from these mutant TTP expression constructs is demonstrated in the western blot shown in Fig. 2C.

Using similar techniques, we identified several additional mutations in conserved residues that completely abrogated binding to both the TNFα and GM-CSF ARE probes. For example, within the Cx3H interval of the cognate hTTP zinc fingers (see Fig. 1), the aromatic amino acid F at cognate positions 126/164 is conserved in all of the vertebrate CCCH family tandem zinc finger proteins shown in Fig. 1. Substitution with Y in either position had little or no effect on TTP binding to the two ARE probes (Figs. 3A and B, lanes 3 and 6). However, when the F at either position was replaced with a non-aromatic amino acid such as N or L, ARE binding activity was abolished (Fig. 3A and B, lanes 4, 5, and 7). The comparable expression of the mutant TTP proteins is documented in the western blot shown in Fig. 3C, and the positions of the cognate F residues that were mutated in this experiment are shown in Fig. 3D.

2. Effect of non-binding mutations on accumulation of TNFα mRNA in cell transfection studies.
We next tested whether the ability of a given mutant to bind to the TNFα and GM-CSF AREs correlated with its ability to promote the degradation of TNFα mRNA in 293 cell transfection studies. As expected from our previous studies (23), co-expression of low concentrations of wild-type TTP with the TNF expression plasmid led to the essentially complete destruction of the full-length TNFα mRNA, with accumulation of the deadenylated form of the transcript at higher TTP concentrations (Fig. 4A, lanes 1-3). However, when similar concentrations of the C124R mutant were co-expressed with the TNFα cDNA, there was an unexpected increase in steady state TNFα mRNA levels, as well as the absence of conversion of the mRNA to the deadenylated form (Fig. 4A, lanes 5 and 6). When data from five similar experiments were averaged after normalization for RNA loading with GAPDH mRNA, as in the middle panel of Fig. 4A, the mean increase in TNFα mRNA accumulation at 0.01 µg and 0.1 µg of transfected C124R mutant TTP DNA was to 200% and 406% of control, respectively. The amount of expressed mutant TTP mRNA that resulted from these transfections is shown in the lower panel of Fig. 4A; as with the wild-type TTP vector, the mutant mRNA levels correlated closely with protein expression (19).

To determine whether the other non-binding mutations in TTP would have a similar effect on TNFα mRNA accumulation, the effect of wild-type TTP expression was compared to mutants of the key cysteine and histidine residues in the TZF domain, including C124H, C124R, H128K, C147R, C162H and H166L, all transfected at 0.1 µg of DNA (Fig. 4B). Compared to the typical effect of wild-type TTP (upper panel, lane 2), in which most of the TNFα mRNA had been truncated to the deadenylated form (see ref. 19), the six non-binding TTP mutants (lanes 3-8) caused the increased accumulation of the full-length TNFα mRNA; after correcting for transfection
efficiency and RNA loading, the increases in TNFα mRNA levels in this experiment were to 470%, 514%, 537%, 430%, 320% and 440% of control, respectively. The levels of the GAPDH mRNA, used as a gel loading control, and of the expressed normal and mutant TTP mRNAs, are indicated in the middle and lower panels, respectively, of Fig. 4B.

Similar increases in the levels of the TNFα mRNA were seen with all other constructs tested that expressed non-binding mutants of TTP, including those that involved mutations in amino acids other than the key cysteine and histidine residues. For example, as shown in Fig. 4C, the phenylalanine mutants at the cognate residues 126/164 within the Cx3H domain that bound the ARE normally were able to promote the degradation of the co-transfected TNFα mRNA (Figs. 4C, lanes 1-3, 6,7,10,11), whereas the non-binding mutants of the same residues also promoted the accumulation of the fully adenylated TNFα transcript (Fig. 4C, lanes 4,5,8,9).

This increased accumulation of the TNF mRNA was seen with all non-binding mutants tested. The effects of all of the mutations tested on ARE binding in the cell-free assay, as well as their ability to promote TNFα mRNA breakdown in transfected 293 cells, are summarized in Table 1. To date, there has been a perfect correlation between the ability of a TTP mutant to bind to an ARE probe and its ability to promote TNFα mRNA degradation in the cell transfection experiments. In addition, all of the non-binding mutants tested to date have promoted an increased accumulation of TNFα mRNA (Table 1).

3. Effects of non-binding mutants on the accumulation of full-length TNFα, GM-CSF and IL-3 mRNAs.
One limitation of the previous experiments is the use of an artificially truncated form of the TNFα mRNA, in which a synthetic, 33 residue poly A tail is attached directly to a shortened version of the ARE (19). To address this issue, as well as the specificity of the effect on the TNFα mRNA, we co-expressed the wild-type and mutant forms of TTP with three full-length mRNAs containing well-known class II AREs, those encoding TNFα, GM-CSF and IL-3 (Fig. 5). 293 cells do not express detectable levels of these mRNAs. As seen with the truncated construct, co-expression of mutant TTP with a full-length TNFα mRNA resulted in its increased accumulation (Fig. 5A, top panel). Co-expression of a full-length GM-CSF mRNA with wild-type TTP caused the concentration-dependent disappearance of the GM-CSF mRNA (Fig. 5B, top panel, lanes 3-5); however, when the GM-CSF mRNA was co-expressed with the TTP zinc finger mutant C124R, there were markedly increased accumulations of GM-CSF mRNA that corresponded to the increased amount of transfected mutant TTP DNA (Fig. 5B, lanes 6-8).

The results obtained from co-expression of full-length IL-3 mRNA with wild-type TTP and its zinc finger mutant C124R were very similar to those described for GM-CSF (Fig. 5C). At 0.01-1 µg of co-transfected TTP DNA, the IL-3 mRNA almost completely disappeared (Fig.5C, lanes 3-5). When the cells were co-transfected with increasing amounts (0.01 - 0.1 µg) of the C124R mutant form of TTP (Fig. 5C, lanes 6-8), there was a concentration-dependent increase in the accumulation of the IL-3 mRNA. In Figs. 5A-C, the expression of GAPDH mRNA, and of the wild-type and mutant TTP mRNAs, are shown in the middle and lower panels, respectively.

4. Effect of non-binding mutant TTP on TNFα mRNA turnover.
To begin to address the mechanism by which the non-binding mutants caused increased accumulation of ARE-containing mRNAs, we determined whether the presence of a non-binding mutant TTP affected the stability of the TNF\(\alpha\) mRNA in 293 cells by transfecting 1 \(\mu\)g of DNA of the CMV.TNF\(\alpha\) vector into the cells, then exposing them to 0.01 mg/ml actinomycin D 48 h later. TNF\(\alpha\) mRNA levels were quantitated at 0, 4, and 8 h after actinomycin D addition (Fig. 6). Phosphorimager values for TNF\(\alpha\) mRNA levels were corrected for gel loading by Phosphorimager analysis of cyclophyllin mRNA (lower panel of Fig. 6), and for transfection efficiency by a co-transfected human growth hormone plasmid (19).

Under these conditions in 293 cells, the TNF\(\alpha\) mRNA was relatively stable, with a half-life of approximately 9h (Fig. 6, lanes 1-3). In the presence of the co-expressed C124R TTP mutant, there was a marked increase in steady-state levels of TNF\(\alpha\) mRNA to approximately 3-fold of control (mean of two experiments; Fig. 6, compare lane 4 to lane 1). When these cells were exposed to actinomycin D, the TNF\(\alpha\) mRNA half-life was increased three-fold to approximately 28h (Fig. 6, lanes 4-6). These data indicate that expression of the non-binding TTP mutant caused an increase in the stability of the TNF\(\alpha\) mRNA after actinomycin D treatment.

4. Effect of the C124R mutant on the ability of wild-type TTP to promote TNF\(\alpha\) mRNA degradation.

To evaluate the influence of the non-binding TTP mutants on the effects of wild-type TTP, we used a TTP-green fluorescent protein fusion protein (TTP-GFP) that can stimulate normally the breakdown of TNF\(\alpha\) mRNA in the 293 cell transfection system, and can be crosslinked normally to a TNF\(\alpha\) mRNA ARE probe (19). The larger size of the resulting fusion mRNA and protein allows
them to be distinguished from the corresponding mutant TTP mRNA and protein. In this experiment, co-tranfection of the TNFα expression construct with 0.1 µg of the C124R mutant resulted in the usual marked increase in TNFα mRNA expression (Fig. 7, compare lane 2 to lane 1), and expression of wild-type TTP had its usual dose-dependent effect to degrade the TNFα mRNA, with accumulation of the deadenylated form of the mRNA (Fig. 7, lanes 3-5). The TTP-GFP fusion protein had a similar effect to native TTP (Fig. 7, lanes 6-8). When either wild-type TTP (hTTP) or the TTP-GFP fusion protein (hTTP-G) was co-expressed with 0.1 µg of the C124R mutant construct, there were clear increases in the accumulation of TNFα mRNA (Fig. 7, lanes 9-14), at all concentrations of TTP and TTP-GFP tested. For example, compare lane 9 to lane 3, and lane 12 to lane 6. Thus, the mutant C124R construct appeared to inhibit the TTP-induced destruction of the TNFα mRNA, even though it did not interfere significantly with the protective effect of higher concentrations of TTP to increase the accumulation of the shorter, deadenylated TNFα mRNA species (see ref. 19). The expression of the various TTP mRNAs in this experiment is shown in the lower panel of Fig. 7; note that the wild-type and mutant TTP mRNAs are of identical size and are thus superimposed in lanes 9-11.

We also tested whether co-expression of the C124 mutant with the wild-type protein in the same cells interfered with binding to the ARE probe (Fig. 8). In this situation, at concentrations (0.05 to 0.1 µg of DNA) that were optimal for causing increased TNFα mRNA accumulation in these cells, there was no apparent interference by the mutant protein on the binding of wild-type TTP to the ARE probe (Fig. 8, lanes 4, 5), assayed in this case by UV crosslinking of the protein to the ARE probe. At higher concentrations of C124R DNA, there was a modest decrease in the binding of wild-type TTP to the ARE probe (Fig. 8, lanes 6, 7). However, when the identical experiment was
performed using the TTP-GFP fusion protein, there was no apparent effect of the TTP mutant to interfere with crosslinking of the fusion protein to the ARE probe (Fig. 8, lanes 8-12).

Discussion

The principal finding of the studies described here is that certain mutant derivatives of TTP that are incapable of binding to the AREs of the TNFα and GM-CSF mRNAs exert an inhibitory effect on the turnover of these mRNAs. It is generally accepted that the destruction of mRNAs containing class II AREs is accomplished by initial deadenylation to essential completion, followed by destruction of the remaining mRNA body (24,25). The inhibitory mutants described in this study appear to inhibit the initial step in this destruction process, that of deadenylation. This is in keeping with previous data indicating that the primary effect of wild-type TTP in this experimental system, and presumably in normal cellular physiology, is to stimulate the initial deadenylation of class II ARE-containing mRNAs (19). The facts that TTP mutants that are incapable of RNA binding not only are ineffective at stimulating breakdown of class II ARE-containing mRNAs, but also appear to protect these from deadenylation and subsequent destruction in intact cells, suggests that strategies directed at discovering factors interacting with the mutants might lead to elucidation of TTP's mode of action.

How might this inhibitory effect of the non-binding mutant forms of TTP be accomplished? One potential mechanism is the formation of heterodimers with wild-type TTP, thus preventing it from binding to the mRNA in a stoichiometric fashion. However, the inhibitory effect of the mutant proteins was observed in the complete absence of transfected or endogenous wild-type TTP; in other words, there was no normal TTP present to inactivate by this means. In addition, there was no
apparent inhibition of TTP binding to the TNFα or GM-CSF mRNA AREs by the mutant proteins in cell-free direct binding and crosslinking assays. A second possible mechanism is suggested by the facts that all of the inhibitory mutants examined were incapable of binding to the TNFα or GM-CSF mRNA ARE, and that, conversely, every known non-binding mutant acts in a similar inhibitory fashion at equivalent amounts of transfected DNA. This means that these mutants could not be inhibiting deadenylation by binding to and blocking the ARE. Instead, the data raise the interesting possibility that the mutant forms of TTP can inhibit mRNA breakdown by interacting with a protein or proteins responsible for promoting or inhibiting this activity. Candidate proteins for this putative interaction include polyA binding proteins, ARE-binding proteins, deadenylating enzymes and others, perhaps in a multimeric complex.

After actinomycin D treatment of 293 cells expressing the TNFα mRNA, the rate (R1) of conversion of the fully adenylated form of TNFα mRNA to its deadenylated intermediate must be appreciably slower than the rate (R2) of degradation of the deadenylated intermediate, since there was no detectable accumulation of this intermediate under these conditions in the absence of TTP. We have calculated the half-life of the TNFα mRNA to be approximately 9 h under these conditions (see Fig. 6). At low concentrations of transfected wild-type TTP DNA (e.g., 5 and 10 ng; see lanes 2 and 3 of Fig. 4), R1 must increase so that it is approximately equal to R2, since neither the fully polyadenylated nor the deadenylated form of TNFα mRNA accumulates significantly. However, at higher concentrations of transfected, wild-type TTP (e.g. 0.1 and 1 µg; see lane 4 of Fig. 4 and lanes 4 and 5 of Fig. 7), the smaller, deadenylated form of TNFα mRNA accumulates, indicating that R1 is significantly greater than R2 under these conditions. This accumulation of the smaller, deadenylated form of the TNFα mRNA was largely unaffected by the presence of any concentration of mutant
TTP (see Fig. 7). These data indicate that the inhibitory forms of TTP had little if any effect on the rate (R2) of degradation of the deadenylated TNF\(\alpha\) mRNA body, which was inhibited in its characteristic fashion by concentrations of wild-type TTP DNA of 0.1 and 1 \(\mu\)g whether the non-binding mutant was present or not. Thus, the effect of the mutant TTP to cause accumulation of the larger, fully adenylated form of TNF\(\alpha\) mRNA appears to be limited to an effect on the rate (R1) of conversion of the adenylated to the deadenylated form of this mRNA. This suggests in turn that the effect of high concentrations of wild-type TTP to cause accumulation of the smaller form of TNF\(\alpha\) mRNA is due to an inhibitory effect on the degradation of the mRNA body, either by binding to and stabilizing the body, or by inhibiting the action of one or more endo- or exonucleases.

Our previous studies have identified TTP and its related proteins as RNA binding proteins, with apparent specificity to the class II ARE, and have established the TZF domain as the domain responsible for the RNA binding. These studies have established the TZF domain as a novel RNA binding motif, and have demonstrated that disruption of the tandem zinc fingers by mutations to the key cysteine or histidine residues completely abrogates RNA binding. The present studies have extended these observations by beginning to establish the primary sequence requirements for the ARE binding activity of this novel motif, particularly as regards replacement of highly conserved residues within the C-C and C-H intervals. These and further studies should help establish the key amino acids within these intervals for binding to TTP’s known physiological targets, the TNF\(\alpha\) and GM-CSF mRNA AREs, as well as perhaps TTP-specific residues that differentiate its binding characteristics from those of other family members. Future studies will continue this exploration of the TZF by examining the effects of the spacing among the key cysteine and histidine residues, as well as the interfinger spacing, all of which are exactly conserved in known members of this protein
family. The ultimate goals will be a description of the structure of this domain bound to its mRNA target, as well as a description of the mechanism by which these proteins promote destabilization of their target mRNAs.

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Footnotes

1. The abbreviations are: TTP, tristetraprolin; TNFα, tumor necrosis factor α; ARE, AU-rich element; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin-3; 3'-UTR, 3'-untranslated region; HGH, human growth hormone; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; b, base(s); bp, base pair(s); BSA, bovine serum albumin.

2. W.S. Lai and P.J. Blackshear, unpublished data.

References


Figure legends

Figure 1. Alignment of the TZF domain from known vertebrate CCCH TZF proteins.

The figure shows an alignment of the TZF domains from vertebrate TTP, cMG1 and TIS11D proteins. Amino acid residues that are conserved within each of the three major subgroups are shown as a consensus (Cons.) below each subgroup; residues that are conserved among all the proteins are shown at the bottom as an overall consensus. The residues in parentheses refer to conservative substitutions in that position. The small letters in front of each protein name indicate the animal species: h, human; b, bovine; m, mouse, r, rat; x, *Xenopus laevis*. The accession numbers for all proteins are listed in (20) except for bovine TIS11D, which is derived from a conceptual translation of the bovine ESTs AW668956 and AW463156. The letters in bold indicate the key CCCH residues in each finger, and the numbers at the top refer to the residue number in the GenBank RefSeq for human TTP, NP_003398. See the text for further details.

Figure 2. Binding of hTTP and CCCH zinc finger mutant proteins to the TNFα and GM-CSF mRNA AREs.

In A-C, cytosolic extracts were prepared from 293 cells transfected with either vector alone (lane 1, BS+, 5 µg/plate) or constructs expressing hTTP (Wt) and various CCCH zinc finger mutant proteins (C124R, etc, as indicated; lanes 2-8, 0.1 µg DNA/plate, together with 4.9 µg/plate of vector DNA) were prepared as described in Experimental Procedures. For A and B, electrophoretic mobility shift assays were performed with these extracts. Cell extracts (5 µg of protein/lane) were incubated with 2 x 10^5 cpm of a TNFα (A) or a GM-CSF (B) ARE probe. P: Probe alone (RNase
T1 digested). The migration positions of the TTP-probe complexes (TTP), the RNA-protein complexes I, II and III formed with endogenous 293 cell proteins, and the migration position of the free probes (FP) are indicated. In C, cell extracts prepared from 293 cells as described above were analyzed by western blotting using a polyclonal antibody to the epitope tag of the fusion proteins, and the position of epitope-tagged TTP protein is indicated. The positions of molecular weight standards are indicated to the left of the gel.

**Figure 3. Evaluation of point mutants of conserved amino acids in the Cx3H interval of the tandem zinc fingers in the CMV.hTTP.tag construct.**

In A-C, cytosolic extracts of 293 cells transfected with either vector alone (lane 1, BS+, 5 µg of DNA/plate) or constructs expressing hTTP (Wt) and various mutant proteins (F126Y, etc, as indicated; lanes 2-7, 0.1 µg DNA/plate, together with 4.9 µg/plate of vector DNA) were prepared as described in Experimental Procedures. For A and B, electrophoretic mobility shift assays were performed with these extracts. Cell extracts (5 µg of protein/lane) were incubated with 2 x 10^5 cpm of either a TNFα (A) or a GM-CSF (B) ARE probe. The migration positions of the TTP-probe complexes (TTP), the RNA-protein complexes I, II and III formed with endogenous 293 cell proteins, and the migration positions of the free probes (FP) are indicated. In C, cell extracts prepared from 293 cells as described in A and B were analyzed by western blotting, using a polyclonal antibody to the epitope tag of the fusion proteins, and the position of tagged TTP protein is indicated (TTP). The positions of molecular weight standards are indicated to the left of the gel. D represents an alignment of the two CCCH zinc fingers (ZF I and ZF II) from hTTP. The CCCH motifs of both fingers are in bold type. The sequences of the two Cx3H intervals are in larger font.
size. The cognate aromatic amino acids F126 and F164 are conserved in the known vertebrate CCCH TZF proteins shown in Fig. 1.

**Figure 4. Effect of mutations in the zinc finger CCCH motifs of TTP on stability of TNFα mRNA.**

In A - C, CMV.mTNFα (1 µg/plate) was co-transfected into 293 cells with TTP (Wt), or with the CCCH zinc finger mutant protein expression constructs (C124H, etc, as indicated), or with vector alone (BS+). Total cellular RNA was harvested as described in Experimental Procedures. Each gel lane was loaded with 10 µg of total cellular RNA. Electrophoresis and northern hybridization were performed as described in Experimental Procedures. 

**A**, upper panel: RNA from 293 cells co-transfected with 1 µg/plate of a CMV.mTNFα construct encoding b 127-1325 of GenBank accession number X02611, with 33 A residues attached to b1325 (19). Vector or TTP expression constructs were co-transfected as follows: Lane 1: Vector alone (BS+, 4 µg of DNA/plate); lanes 2, 3 and 4: CMV.hTTP.tag (0.005, 0.01 and 0.1 µg/plate, respectively); lanes 5, and 6: C124R (0.01 and 0.1 µg/plate, respectively). For lanes 2-6, vector DNA was also added to make the total co-transfected plasmid DNA equal to 5 µg/plate. 

**B**, upper panel: Accumulation of TNFα mRNA in the presence of other CCCH mutants. Vector and TTP expression constructs were co-transfected as follows: Lane 1: Vector alone (BS+, 4 µg/plate); lanes 2-8: Wild-type CMV.hTTP.tag or CCCH mutant constructs as indicated (0.1 µg of DNA/plate), together with 3.9 µg/plate of vector DNA. In **C.**, CMV.mTNFα (1 µg/plate) was co-transfected into 293 cells with the indicated amount of DNA (in ng per plate of cells) from vectors expressing TTP (Wt), with the mutant protein expression constructs (F126N, etc, as indicated), or with vector alone (BS+). Vector
DNA was added (lanes 2-11) to make the total co-transfected plasmids 4 µg/plate of DNA. In the upper panels of A-C, the northern blots were probed with a 32P-labeled TNFα cDNA, and the two major species of TNFα mRNA present are indicated by the arrows (TNFα). After autoradiography, the blot was reprobed with a 32P-labeled GAPDH cDNA probe (A and B), or a cyclophilin (Cyclo) cDNA probe (C), and the results are shown in the middle panels. Identical filters to those in the upper panels were blotted and probed with a 32P-labeled mouse TTP cDNA, and the amounts of TTP mRNA are shown in the lower panels (TTP). The position of the 18S ribosomal RNA is indicated in each case.

**Figure 5. Effect of the C124R mutation on the accumulation of full-length TNFα, GM-CSF and IL-3 mRNAs.**

Expression constructs containing full-length mouse TNFα (A), mouse GM-CSF (B), and mouse IL-3 (C) cDNAs were co-transfected into 293 cells with wild-type TTP or the zinc finger mutant C124R expression constructs, or with vector alone (BS+), as indicated. Total cellular RNA was harvested as described in Experimental Procedures. Each gel lane was loaded with 10 µg of total cellular RNA. Electrophoresis and northern hybridization were performed as described in Experimental Procedures. A, upper panel: RNA from 293 cells co-transfected with a CMV.TNFα construct encoding b 1-1627 of X02611 (lanes 1 and 2, 5 µg/plate). Vector or TTP expression constructs were co-transfected as follows: Lane 1: Vector alone (BS+, 0.1 µg/plate); lane 2: C124R (0.1 µg/plate). In the upper panel of A, the northern blot was probed with a 32P-labeled mTNFα cDNA probe (bp 127-1200). The position of the 18S ribosomal RNA is indicated. The arrows labeled TNFα indicate the two species of TNFα mRNA discussed in the text. B, upper panel: RNA
from 293 cells co-transfected with CMV.mGM-CSF (1 µg/plate; lanes 2-8) and TTP expression constructs or vector alone. Lane 1: Mock transfection (BS+, 5 µg of DNA/plate); lane 2: Vector alone (BS+, 4 µg/plate); lanes 3-5: CMV.hTTP.tag (0.005, 0.01, and 0.1 µg/plate, respectively); lanes 6-8: C124R (0.005, 0.01 and 0.1 µg/plate, respectively). For lanes 3-8, vector DNA was also added to make the total co-transfected plasmid DNA equal to 5 µg/plate. The northern blot shown in the upper panel was probed with a 32P-labeled mGM-CSF cDNA, and the two major species of GM-CSF mRNA present are indicated by the arrows. C, upper panel: RNA from 293 cells co-transfected with CMV.mIL-3 (1 µg/plate; lanes 2-8) and TTP expression constructs, or vector alone. Lane 1: Mock transfection (BS+, 5 µg of DNA/plate); lane 2: Vector alone (BS+, 4 µg/plate); lanes 3-5: CMV.hTTP.tag (0.01, 0.1 and 1 µg/plate, respectively); lanes 6-8: C124R (0.01, 0.05 and 0.1 µg/plate, respectively). For lanes 3-8, vector DNA was also added to make the total co-transfected plasmid DNA equal to 5 µg/plate. The northern blot shown in the upper panel was probed with a 32P-labeled mIL-3 cDNA. The three arrows in the upper panel indicate the three species of IL-3 mRNA discussed in the text. For A - C, after autoradiography the blots corresponding to the upper panels were stripped and reprobed with a 32P-labeled GAPDH probe, and the results are shown in the middle panels. Identical RNA samples as in the upper panels were blotted and probed with a 32P-labeled mouse TTP cDNA, as shown in the bottom panels of A-C. The position of the 18S ribosomal RNA is indicated in each case. The expressed GAPDH and TTP mRNAs are also indicated by arrows.

Figure 6. Relative stability of TNFα mRNA in the presence of mutant C124R and actinomycin D.
CMV.mTNFα (1 µg/plate) was co-transfected into 293 cells without (-) or with the CCCH zinc finger mutant protein expression construct (+C124H), as indicated. The cells were exposed to actinomycin D (Act D) for the indicated times, after which total cellular RNA was harvested as described in Experimental Procedures. Each gel lane was loaded with 10 µg of total cellular RNA. Electrophoresis and northern hybridization were performed as described in Experimental Procedures. In the upper panel, the blots were probed with a 32P-labeled TNFα cDNA, and the single species of TNFα mRNA present is indicated by the arrows. The position of the 18S ribosomal RNA is indicated. In the lower panel, the same blot was reprobed with a radiolabeled cyclophilin (cyclo) cDNA probe, as indicated.

Figure 7. Effect of adding the inhibitory C124R mutant form of TTP on the ability of low concentrations of wild-type TTP to promote TNFα mRNA degradation.

CMV.mTNFα (1 µg/plate) was co-transfected into 293 cells with CMV.hTTP.tag, or with its C124R mutant, or with CMV.hTTP.EGFP expression constructs, or with vector alone. Total cellular RNA was harvested as described in Experimental Procedures. Each gel lane was loaded with 10 µg of total cellular RNA. Electrophoresis and northern hybridization were performed as described in Experimental Procedures. Vector or TTP expression constructs were co-transfected as follows: Lane 1: Vector alone (BS+, 4 µg of DNA/plate); lane 2: TTP mutant C124R (0.1 µg/plate); lanes 3-5, 9-11: Wild-type CMV.hTTP.tag (hTTP) as indicated (0.01 — 0.1 µg of DNA/plate); lanes 6-8, 12-14: CMV.hTTP.EGFP (hTTP-G) as indicated (0.01 — 0.1 µg of DNA/plate); C124R (0.1 µg/plate) was also added to the plates for lanes 9-14. Vector DNA was added to make the total co-transfected plasmid DNA equal to 5 µg/plate in lanes 2-14. The northern blots shown in the upper panel were
probed with a $^{32}$P-labeled TNFα cDNA probe and a $^{32}$P-labeled cyclophilin cDNA probe, and the two major species of TNFα mRNA present are indicated by the arrows. The cyclophilin mRNA is also indicated. An identical filter as in the upper panel was blotted and probed simultaneously with a $^{32}$P-labeled mouse TTP cDNA, and the results are shown in the lower panel. The expressed TTP mRNA (hTTP) and the expressed TTP-GFP fusion mRNA (hTTP-G) are indicated. Note that in lanes 9-11, the wild-type TTP and mutant C124R mRNA are superimposed; however, in lanes 12-14 the wild-type TTP fusion protein mRNA (hTTP-G) and the mutant C124R TTP mRNA (hTTP) are widely separated in the blot. The position of the 18S ribosomal RNA is indicated in each case.

Figure 8. Effect of the inhibitory constructs on ARE binding of wild-type TTP and a fusion protein consisting of TTP and a modified version of the green fluorescent protein (TTP-GFP).

Shown are the results of UV cross-linking assays with a TNFα ARE probe. Cytosolic extracts of 293 cells transfected with either vector alone (BS+) or constructs expressing wild-type TTP (hTTP), or its C124R mutant, or a TTP-GFP fusion protein (hTTP-GFP) were prepared as described in Experimental Procedures. A $^{32}$P-labeled mTNFα (bp 1281-1350 of GenBank accession number X02611) ARE probe (1.5 x 10^6 cpm/sample) was then incubated with extracts (5 µg of protein). UV cross-linking and RNase digestion were performed as described in Experimental Procedures. Lane 1 (BS+): Extract from 293 cells transfected with 5 µg/plate of vector DNA. Lane 2 (C124R): Extract from 293 cells transfected with 1 µg/plate of plasmid DNA from C124R. Lanes 3-7: Extracts from 293 cells transfected with 1 µg/plate of plasmid DNA from CMV.hTTP.tag. Lanes 8-12: Extracts from 293 cells transfected with 1 µg/plate of plasmid DNA from
CMV.hTTP.GFP. Lanes 4-7, and 9-12: Different amounts of C124R (µg/plate) were also added to each plate as indicated. Vector DNA was added to make the total transfected DNA 5 µg/plate. The RNA-protein complexes were resolved by SDS-PAGE (12% gel) followed by autoradiography. The positions of molecular weight standards, and the protein-probe complexes formed with either TTP or TTP-GFP, are indicated.
Table 1. Summary of point mutant effects on TTP binding to an ARE probe and destabilizing an ARE-containing mRNA.

These data summarize the results from 3-4 individual experiments. Interval represents the distance between each of two key amino acids in the two CCCH motifs, and X represents any amino acid. Amino acid change refers to the results of mutations encoding conserved amino acids of the CCCH motifs, as illustrated in Fig. 1. ARE binding means the ability of expressed hTTP mutants to bind to a GM-CSF mRNA ARE probe, as assessed by gel mobility shift assays. Binding activity of wild-type TTP is indicated by ++++, whereas no binding activity is indicated by 0. Decrease TNFα mRNA represents the ability of a mutant TTP construct to cause breakdown of the TNFα mRNA in co-transfection experiments in 293 cells, indicated as 0 (No effect) to ++++ (equivalent to wild-type TTP). Increased TNFα mRNA refers to the ability of a given mutant to cause an increase in the steady state concentration of TNFα or other ARE-containing mRNAs in the 293 cell co-transfection studies.
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Fig 1
A

B

C

Fig 2
Fig 3

A

B

C

D

ZF I: CRTFSERGCKYGAKCQFAH
ZF II: CRKFLQGCPYGSRCHFTH
Fig 5
Fig 6
Interactions of CCCH zinc-finger proteins with mRNA. Non-binding tristetraprolin mutants exert an inhibitory effect on degradation of AU-rich element containing mRNAs

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