mRNA Degradation: an Important Process in Controlling Gene Expression

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Tristetraprolin and other CCCH tandem zinc-finger proteins in the regulation of mRNA turnover

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Abstract

The tristetraprolin (TTP) family of CCCH tandem zinc-finger proteins is composed of three known members in mammals, with a fourth member recently identified in frogs and fish. Although TTP was first cloned more than 10 years ago as a growth factor-induced gene, a physiological function for the protein has been discovered only within the last few years. TTP is now known to bind to so-called class II AU-rich elements within the mRNAs that encode tumour necrosis factor-α and granulocyte/macrophage colony-stimulating factor. In both cases, this binding results in destabilization of the mRNA and decreased secretion of the protein. Recent evidence suggests that TTP can accomplish this accelerated mRNA degradation by first promoting removal of the polyadenylated tail from the mRNA (deadenylation). In functional assays in cells, the other family members have similar activities, but are expressed differently in tissues and in response to stimuli, suggesting that they may control the stability of mRNAs under different circumstances from those in which TTP affects mRNA. All of these proteins are phosphoproteins and nucleocytoplasmic shuttling proteins, suggesting that their activities can be regulated in ways other than regulating gene transcription. Together, the TTP family members should be capable of complex regulation of short-lived mRNAs containing this type of AU-rich instability motif.

Key words: AU-rich element, cytokine, deadenylation.

Abbreviations used: ARE, AU-rich element; BRF, butyrate-response factor; ERF, EGF-response factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; GOS24, G0/G1 switch regulatory gene 24; KO, knockout; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NUP475, nuclear protein 475; poly(A)$^+$, polyadenylated; TIS11, TPA-induced sequence 11; TNF, tumour necrosis factor; TTP, tristetraprolin; WT, wild-type; ZFP, zinc-finger protein.

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Our laboratory first became involved in the study of tristetraprolin (TTP) and other CCCH tandem zinc-finger proteins (ZFPs) more than 10 years ago. The object of a study carried out by Lai et al. [1] was to develop a screen for genes that were rapidly turned on at the transcriptional level in response to physiological concentrations of insulin. This was done by screening an insulin-sensitive fibroblast library using cDNAs from control cells and cells stimulated by insulin. One of the genes that was induced rapidly was a sequence encoding an unknown, hypothetical protein that was named TTP [1]. After the insulin-responsive cells had been starved of serum overnight, TTP mRNA expression was barely de-

Owing to unforeseen circumstances, this Colloquium was cancelled after the articles presented here were written.

tectable. However, within 10 min of the addition of physiological concentrations of insulin, there was detectable accumulation of TTP mRNA. These levels peaked at approx. 30–45 min, before decreasing rapidly to reach near-baseline values by 2 h. Thus this novel gene appeared to display the characteristics of a so-called immediate early response gene, whose rapid, massive and transient induction occurred in response to insulin. Similar rapid and transient responses could be achieved in fibroblasts in response to serum, growth factors, tumour-promoting phorbol esters and other extracellular agonists, but not in response to agents that acted by elevating cAMP levels. More recent work has shown that TTP can be induced with similar kinetics in macrophages stimulated with lipopolysaccharide (LPS) or tumour necrosis factor (TNF)-α with similar rapid on-and-off induction characteristics [2]. The stimulation of mRNA accumulation was shown to be largely at the level of transcription, with no apparent evidence for marked regulation of mRNA stability.

In completely independent work, a partial clone of this cDNA had been identified earlier by Herschman and colleagues [3] in a screen for phorbol ester-responsive genes; they later published the complete corrected sequence [4]. It was also cloned, nearly simultaneously, by DuBois, then at Johns Hopkins University, Baltimore, MD, U.S.A., who identified it as a serum-induced gene [5]. In all three cases, the cDNA was found to encode TTP, although these authors and others have called it by a variety of aliases including TIS11 (TPA-induced sequence 11), NUP475 (nuclear protein 475) and GOS24 (G0/G1 switch regulatory gene 24). We called it TTP because of three characteristic PPPPG motifs within the primary sequence of the predicted protein, although we had no idea of the function of the protein at that time.

Further work evaluated the promoter characteristics of this gene, since we were interested in its rapid induction by insulin and other agents. By careful analysis of both the promoter and intron, it was determined that there was a number of both known and unknown transcription-factor-binding sites within the proximal promoter of the gene, which appeared to be responsible for most of the rapid induction of the gene by serum [6]. For example, there was an early growth response gene product 1-binding site within 79 bp of the transcription start site, an AP2 (activator protein 2)- like binding site still further downstream, as well as a novel transcription factor binding site which we have called TTP promoter element 1. In all cases, these elements behaved like classical enhancer elements in that, for example, they could be placed into non-serum-inducible genes to confer serum inducibility to those genes, and they could be reversed and placed elsewhere in the gene for serum-inducible behaviour. It was also found that the single intron was required for optimal serum induction [7]; in addition, it appeared that the presence of the intron in its characteristic location, as well as internal promoter elements, were responsible for much of the serum-inducibility of this gene.

Two other cDNAs were cloned in the early 1990s whose predicted protein sequences resembled TTP, particularly in the tandem zinc-finger domains. One was cloned from a rat intestinal epithelial cell library, as a gene that was responsive to epidermal growth factor, and it was originally named cMG1 by Brown and colleagues [8]. To my knowledge, Brown and colleagues were the first to note the internal repeat sequence that is now referred to as the tandem zinc-finger domain, in both cMG1 and in the partial clone of TTP previously described by Herschman and colleagues [3]. As stated in [8], "... it is noteworthy that each of the repeated elements in TIS11 and cMG1 contains three cysteines and one histidine residue, suggesting a possible co-ordination of these four amino acids with a metal ion in a manner analogous to zinc finger structures." The second additional family member was cloned by the Herschman group in a study that sought to identify cDNAs with similar tandem zinc-finger domains, and they called this TIS11d [9]. The protein translated from their original TIS11d sequence (GenBank® accession number M58564.1) was truncated in the N-terminus and had a reading frame shift in the C-terminus when compared with the current human protein RefSeq (NP_008818) [10]; however, the current mouse RefSeq (NP_031591.1) does not yet reflect these changes to both ends of the protein.

To date, these three proteins are the only known members of the mammalian family of CCCH tandem ZFPs. A fourth family member (and several closely related variants [11]) has been cloned from frogs [10] and fish [12], and this contains not only the characteristic CCCH tandem zinc fingers, but also two more degenerate fingers of this type; this protein is highly expressed in oocytes and eggs in these species, and seems to be restricted to the maternal mRNA pool. The cor-

Table 1

Current nomenclature of human CCCH tandem ZFPs

Gene name	Protein aliases	cDNA RefSeq	Protein RefSeq	Chromosome	OMIM#
ZFP36	TTP, TIS11, GOS24, NUP475	NM_003407	NP_003398	19q13.1	*190700
ZFP36L1	cMG1, TIS11b, ERF1, BRF1, Berg36	NM_004926	NP_004917	14q22–24	*601064
ZFP36L2	TIS11d, ERF2, BRF2	NM_006887	NP_008818	2p22.3–p2	None yet

responding fourth member is not thought to have been identified in mammalian systems.

Within the past year, the HUGO Gene Nomenclature Committee has tried to make the nomenclature of these genes and their encoded proteins more systematic. When we first cloned the human orthologue of mouse TTP, and identified the chromosomal location of the mouse and human genes, the gene was called *ZFP36* in man (*Zfp36* in mouse) [13]. In an attempt to make clear that there were close primary sequence relationships among the three mammalian proteins, the HUGO Gene Nomenclature Committee has recently formalized the names of the human genes as *ZFP36* and *ZFP36L1* (*ZFP36-like 1*) for cMG1 [also known as Berg36, TIS11b, ERF (EGF-response factor) 1 and BRF (butyrate-response factor 1], and *ZFP36L2* for TIS11d (also known as ERF2 and BRF2) (Table 1). The corresponding murine versions of these gene names have also been accepted by the Mouse Genome Database as *Zfp36*, *Zfp36l1* and *Zfp36l2* respectively. The proteins will be referred to here as TTP, ZFP36L1 and ZFP36L2.

Several interesting features are now known about the TTP protein. For example, the protein could be localized to both nuclear and cytosolic compartments in fibroblasts, and its translocation from the nucleus to the cytosol could be accomplished rapidly, i.e. within 5 min, in response to stimuli such as serum, other polypeptide growth factors and phorbol esters, but not by agents that elevate cAMP levels [14]. Phosphorylation of the protein in intact cells, again stimulated by the same agents that stimulated its transcription, was documented, and it was shown that at least one of the sites was Ser^{220} in the mouse sequence, a characteristic mitogen-activated protein (MAP) kinase site that was indeed phosphorylated by MAP kinase both in intact cells and in cell-free systems [15]. When similar experiments were performed in cells expressing a Ser220Ala mutation in mouse TTP, there was a clear loss of SDS/PAGE electrophoretic mobility shift with the mutation; however, this mutation did not seem to affect the ability of extracellular agonists to promote nuclear to cytosolic translocation. Nonetheless, that study identified TTP as a phosphoprotein, predominantly in the form of phosphoserine, and since its phosphorylation status was changed rapidly in response to activators of MAP kinase, such as growth factors and phorbol esters, the results raised the interesting possibility that one or more aspects of the protein's function might be controlled by reversible protein phosphorylation.

The function of the protein began to become apparent when TTP knockout (KO) mice were generated [16]. These animals appeared normal at birth, but within a few months developed a characteristic systemic phenotype that included loss of both weight and body fat, severe polyarticular erosive arthritis and myeloid hyperplasia, both within and outside the bone marrow. They also developed alopecia, dermatitis, conjunctivitis and autoimmunity. Animals from both sexes were occasionally fertile, but no litters have been successfully raised by TTP-deficient mothers. Although we were unable to measure levels of TNF-α in the blood of these animals at that time, the nature of the systemic syndrome suggested the possibility that much of the phenotype was due to a circulating effective excess of TNF-α. This was suggested by previous studies in which exogenous TNF-α, administered by injection or transgenesis, had caused similar weight loss and arthritis syndromes [17,18].

To evaluate this possibility, KO mice and their control littermates were genotyped as soon as possible after birth, and then injected weekly with a hamster anti-mouse monoclonal antibody, kindly provided by Bob Schreiber of Washington University, St. Louis, MO, U.S.A. [19]. These injections completely prevented the weight loss that occurred in the KO animals during the first 10 weeks or so of life, and also prevented the development of the polyarticular erosive arthritis that was characteristic of the syndrome [16]. Other

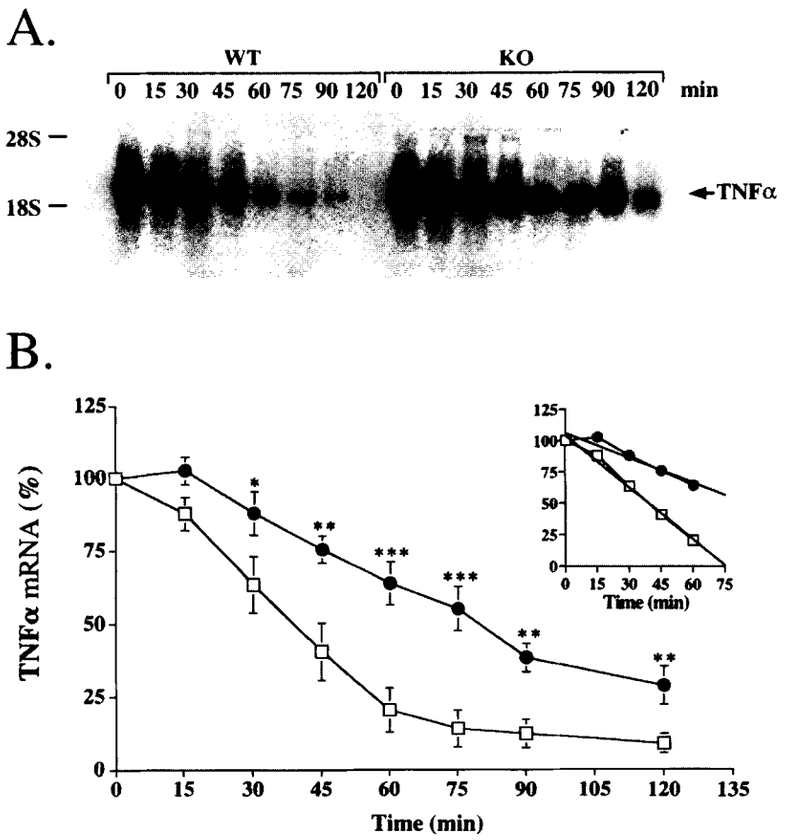
aspects of the phenotype were also prevented. This study suggested strongly that the systemic syndrome was caused, at least in part, by the effective elevation of TNF-α in the circulation, thus identifying TTP as a possible regulator of TNF-α biosynthesis, secretion or turnover.

The mechanism of this effective TNF-α elevation was later partially elucidated in a study which demonstrated that whole bone marrow transplantation into *Rag2* (recombination activating gene) (−/−) immune-deficient mice could completely recapitulate the TTP deficiency syndrome, although after a pronounced lag of nearly 6 months [20]. This late recapitulation suggested that the transfer of the syndrome might not be due to lymphocytes, which are typically repopulated within weeks of a whole bone marrow transplant, but instead might be due to the transplantation of progenitors of other haematopoietic lineages such as monocytes or macrophages. Therefore macrophages from the KO animals were tested for their ability to secrete TNF-α. The critical finding was that macrophages isolated from three different sites (cultured from bone marrow pro-

Figure 1

Effect of TTP deficiency on the stability of TNF-α mRNA in bone-marrow derived macrophages

(**A**) A Northern blot demonstrating the steady-state levels of TNF-α mRNA (TNF-α) in macrophages derived from a pair of littermates, either WT or TTP KO. The cells were stimulated with LPS for 4 h, then treated with actinomycin D at 0 min. The TNF-α mRNA levels were then monitored at intervals for 120 min. (**B**) A graphic representation of the same type of experiment replicated in six mice per group; values are means±S.E.M. of relative mRNA levels: □, WT; ●, KO. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ when comparing the two means at each time point using Student's *t* test. The inset graph represents a linear regression analysis of the same data. The calculated average half-lives of the TNF-α mRNA were 39 min for WT and 85 min for KO in this experiment. Reprinted with minor modifications with permission from Carballo, E., Lai, W. S. and Blackshear, P. J. (1998). 'Feed back inhibition of macrophage humor necrosis factor-alpha production by tristetraprolin' Science, **281**, 1001–1005. © 1998 American Association for the Advancement of Science.



genitors, from the peritoneum and from foetal liver before the onset of the inflammatory syndrome) all revealed markedly increased secretion of TNF-α in response to different concentrations of LPS [20]. This suggested that the primary abnormality leading to the apparent systemic elevation of circulating TNF-α in the TTP KO mice was hypersecretion of TNF-α from the TTP-deficient macrophages and perhaps other cells. An important finding of these studies was that the enhanced levels of TTP secretion were accompanied by increased TNF-α mRNA levels, suggesting that the TTP effect might occur at the level of TNF-α gene transcription or mRNA stability.

In a critical experiment, it was demonstrated that TNF-α mRNA from bone marrow-derived macrophages from the KO animals exhibited a significantly decreased turnover rate compared with TNF-α mRNA from the control animals [2] (Figure 1). These studies were conducted in bone marrow-derived macrophages after stimulation with LPS and inhibition of transcription by actinomycin D, and provided firm evidence that at least one of the molecular abnormalities in the cells derived from the TTP-deficient mice was enhanced stability of the TNF-α mRNA. This led to elevated steady-state levels of the mRNA as well as to increased TNF-α secretion. In parallel studies on transcription of the TNF-α gene, we were unable to document a specific effect of TTP to regulate transcription, although it clearly exhibited transcriptional squelching activity at only modest concentrations of transfected DNA [2].

The same study also investigated the possibility that TTP might be able to bind directly to the TNF-α mRNA, leading in some way to its instability. Using both RNA cross-linking and gel mobility shift techniques, it was found that TTP bound to the so-called class II AU-rich element (ARE) within the 3′ untranslated region of the TNF-α mRNA, a motif known to be involved in TNF-α mRNA instability [2]. A single mutation of only one of the key cysteine or histidine residues within either of the putative zinc fingers prevented this binding. Thus, the binding site for TTP within the mRNA was identified as the ARE, and the RNA-binding site in the protein was identified as the tandem zinc-finger domain. Our working hypothesis was therefore that TTP was an ARE-binding protein that in some way led to mRNA instability. It joined a number of other known ARE-binding proteins, most of which had been shown previously to promote the stability, rather than the instability, of the mRNA to which they were bound.

A number of other mRNAs containing characteristic class II AREs, including that encoding granulocyte/macrophage colony-stimulating factor (GM-CSF), were investigated. It was shown in cultured bone marrow-derived stromal cells that the GM-CSF mRNA was greatly stabilized in cells from the TTP KO mice, compared with cells from the wild-type (WT) animals [21]. This effect was seen not only in the TTP-deficient cells, but also in cells derived from mice that were deficient in TTP and both TNF receptors, thus removing the possibility that excess TNF-α from the TTP KO was stimulating GM-CSF production. It was concluded from these studies that another physiological function of TTP was to regulate the stability of GM-CSF mRNA. It was speculated that the severe myeloid hyperplasia in the TTP-deficient animals might be partly due to a chronic excess of GM-CSF; this aspect of the phenotype became particularly evident in the mice that lacked TTP and both types of TNF receptors, which had longer lifespans and better fertility than the original TTP-deficient mice [22]. This possibility is being investigated at present by interbreeding these triple KO mice with mice in which the GM-CSF gene has been knocked out.

The GM-CSF experiments [21] were critical to our evolving understanding of the mechanism of TTP action. As shown in Figure 2, the GM-CSF mRNA from the WT stromal cells existed as two characteristic species of roughly equal intensity on Northern blots, differing by approx. 200 bp in size; this had been noted previously in other cell types [23,24]. This is in marked contrast with the TNF-α mRNA shown in Figure 1, which existed as a single mRNA species in Northern blots from both WT and KO mice. After the addition of actinomycin D, both species of GM-CSF mRNA decayed rapidly and in parallel in stromal cells from the WT animals. However, in cells from the TTP KO animals, only a single predominant mRNA species was present, the larger of the two mRNAs. Strikingly, it had no detectable half-life of turnover after actinomycin D addition (Figure 2). This suggested that the lower band in the WT cells was the deadenylated mRNA 'body', whereas the upper band contained the full-length polyadenylated [poly(A)$^+$] tail, and that the lack of TTP was preventing removal of the poly(A)$^+$ tail and turnover of the message. This was confirmed by experiments with the RNase H

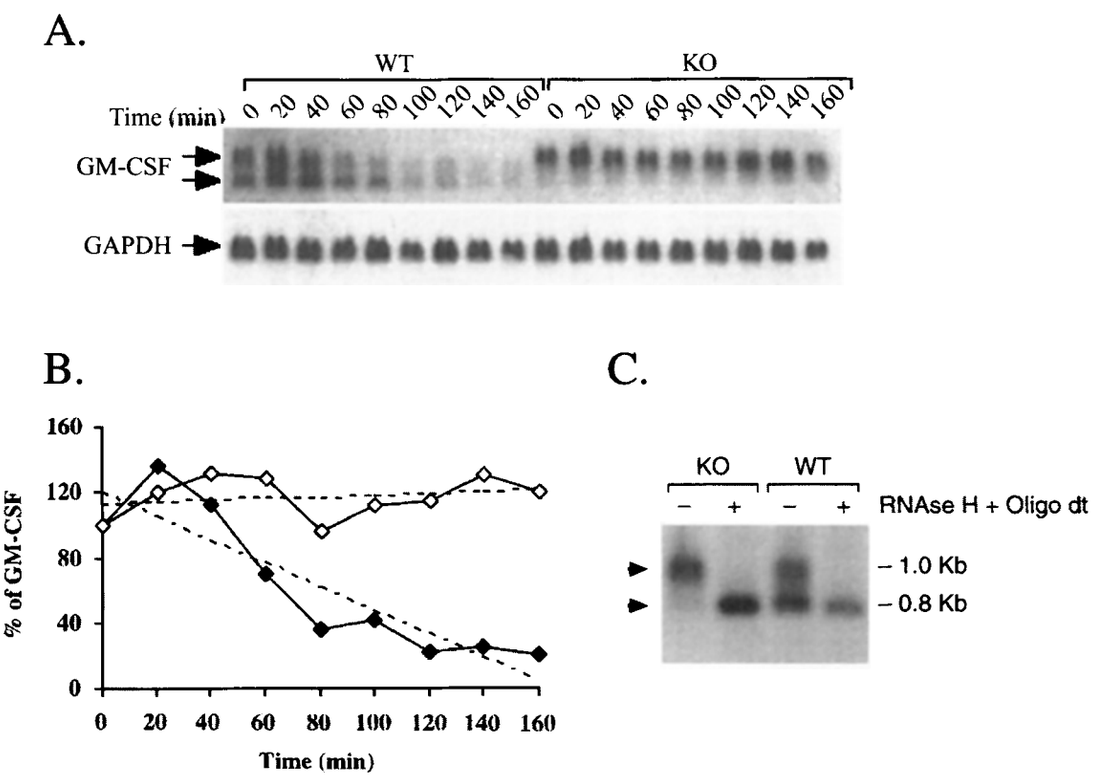
and oligo(dT) (Figure 2), in which the lower band was shown to be the mRNA body minus the poly(A)$^+$ tail, whereas the upper band was the mRNA with the full poly(A)$^+$ tail. Thus, at least in this system, TTP deficiency appeared to prevent deadenylation of this specific mRNA. Since deadenylation has long been thought to be the rate-limiting step in mRNA degradation in vertebrates [25–27], it seemed likely that the reason for the mRNA stability in the TTP KO cells was the failure of TTP to bind to the mRNA and promote first the deadenylation of the message and then its complete degradation. Conversely, this suggested that TTP's primary mode of action was to first bind to the class II ARE in mRNAs, such as those encoding TNF-α or GM-CSF, and then somehow promote the deadenylation of the message and its more rapid destruction.

Thus, our working hypothesis at present is that these proteins represent a class of ARE-binding and deadenylation-promoting proteins. More recently, in transfection studies involving human HEK-293 cells that lack TTP and both of its paralogues, it was shown that TTP could promote the deadenylation and destruction of mRNAs such as those coding for TNF-α, GM-CSF and interleukin-3, and that this could occur in response to transfection of the other two family members as well [28]. The related proteins are often expressed in different cells and tissues compared with TTP, and with different modes of regulation in tissues and in cultured cells, sug-

Figure 2

Effect of TTP deficiency on GM-CSF mRNA stability in bone marrow-derived stromal cells

(**A**) A Northern blot of total cellular RNA probed with either a GM-CSF probe or a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as indicated. The RNA was taken from bone marrow-derived stromal cells from WT or KO mice, as indicated, after the cells had been stimulated for 2 h with LPS and then treated with actinomycin D at 0 min. RNA was then prepared from the cells at intervals for 160 min. Note the two species of GM-CSF mRNA (arrows) in the RNA taken from the WT cells, and the single species in the RNA taken from the KO cells. (**B**) Quantitative data from (**A**). WT RNA values, ◆; KO RNA values, ◇. Broken lines indicate linear regressions of the two data sets. The calculated half-life of the GM-CSF mRNA was 99 min in the control cells but essentially infinity in the KO cells. (**C**) An experiment in which total cellular RNA from either KO or WT cells, equivalent to the samples from 0 min in (**A**), was subjected to treatment with RNase H and oligo(dT) as indicated. This results in removal of the poly(A)$^+$ tail. The upper band of 1.0 kb represents the fully poly(A)$^+$ GM-CSF mRNA; the lower band of 0.8 kb represents the deadenylated mRNA. From Carballo, E., Lai, W. S. and Blackshear, P. J. (2000) 'Evidence that tristetraprolin (TTP) is a physiological regulator of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA deadenylation and stability' Blood **95**, 1891–1899. © American Society of Haematology. Used by permission.



gesting the possibility that they all play some role in conferring message instability, but in ways that are specific to the cell types and patterns of expression under consideration.

Much of our current effort is devoted to unravelling the actual mechanism of how these proteins promote deadenylation and mRNA instability, as well as how these processes are regulated. For example, it is likely that the phosphorylation state of the protein plays an important role in some aspect of its function, and we have demonstrated recently that the completely dephosphorylated protein exhibits enhanced avidity of binding to an ARE-containing mRNA, which suggests the possibility that phosphorylation is important for regulating mRNA-binding affinity in cells [29]. Similarly, it was demonstrated recently that all members of the family contain functional nuclear export sequences that can translocate the proteins from the nucleus to the cytosol, as well as less well-defined nuclear import sequences [30]. Since we had shown previously that this import and export occurs on a very short timescale in response to extracellular stimuli [13], it will be very important to determine whether the effects of modifications of the protein, for example its phosphorylation state, lead to changes in its subcellular localization. Conversely, it will be of great interest to determine how these changes in subcellular localization affect the proteins' function. Finally, we are extremely interested in understanding the molecular details of the proteins' binding to mRNA and the mechanism by which they stimulate mRNA deadenylation. For this purpose, we have recently begun a systematic mutagenesis approach to the tandem zinc-finger domain, and many conserved sites that cannot be altered without the loss of mRNA-binding affinity were recently demonstrated [31]. It was also shown that some of these non-binding mutants exhibit a 'dominant negative' effect on mRNA stability, and that their co-transfection leads to enhanced accumulation of messages, such as TNF-α mRNA, in HEK-293 cells [31]. More recently, we have developed a cell-free deadenylation system that is TTP-responsive, which is currently being used to explore some of the potential mechanisms by which these interactions and subsequent mRNA destruction occur.

By following the results from our initial identification of an insulin-induced gene, unexpected aspects of the function of the encoded protein were discovered by going from the gene to KO mice and back to the protein. The interesting potential mechanisms of action of TTP and related proteins suggested by our results have generated a large number of ongoing studies by our laboratory and others. In addition, the medical relevance of TNF-α and GM-CSF, as well as the possibility that other mRNAs still to be discovered are regulated by these genes, have led to interest among pharmaceutical companies in using these newly described biochemical pathways to develop drugs that affect TNF-α and GM-CSF production. I predict that within the next 10 or 15 years a great deal more will be learned about all aspects of the structure and function of this fascinating family of proteins.

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AU-rich element-mediated translational control: complexity and multiple activities of *trans*-activating factors

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Abstract

Tumour necrosis factor (TNF)-α mRNA contains an AU-rich element (ARE) in its 3′ untranslated region (3′UTR), which determines its half-life and translational efficiency. In unstimulated macrophages, TNF-α mRNA is repressed translationally, and becomes efficiently translated upon cell activation. Gel retardation experiments and screening of a macrophage cDNA expression library with the TNF-α ARE allowed the identification of TIA-1-related protein (TIAR), T-cell intracellular antigen-1 (TIA-1) and tristetraprolin (TTP) as TNF-α ARE-binding proteins. Whereas TIAR and TIA-1 bind the TNF-α ARE independently of the activation state of macrophages, the TTP–ARE complex is detectable upon stimulation with lipopolysaccharide (LPS). Moreover, treatment of LPS-induced macrophage extracts with phosphatase significantly abrogates TTP binding to the TNF-α ARE, indicating that TTP phosphorylation is required for ARE binding. Carballo, Lai and Blackshear [(1998) Science **281**, 1001–1005] showed that TTP was a TNF-α mRNA destabilizer. In contrast, TIA-1, and most probably TIAR, acts as a TNF-α mRNA translational silencer. A two-hybrid screening with TIAR and TIA-1 revealed the capacity of these proteins to interact with other RNA-binding proteins. Interestingly, TIAR and TIA-1 are not engaged in the same interaction, indicating for the first time that TIAR and TIA-1 can be functionally distinct. These findings also suggest that ARE-binding proteins interact with RNA as multimeric complexes, which might define their function and their sequence specificity.

Key words: MAP-kinase-activated protein kinase-2, TIA-1, TIAR, TTP.

Abbreviations used: 3′UTR, 3′ untranslated region; ARE: AU-rich element; AUF1, AU binding factor 1; Elav, embryonic lethal abnormal vision; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; Hel-N1, human Elav-line neuronal protein 1; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MK2, MAP-kinase-activated protein kinase-2; poly(A)$^+$, polyadenylated; RRM, RNA recognition motif; TIA-1, T-cell intracellular antigen-1; TIAR, TIA-1-related protein; TNF, tumour necrosis factor; TTP, tristetraprolin.

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AU-rich elements (AREs): a family of RNA *determinants involved in post-transcriptional regulatory processes*

AREs were first discovered in the 3′ untranslated region (3′UTR) of several cytokine and oncoprotein genes [1]. These elements are composed of a variable number of copies of the AUUUA pentamer or UUAUUUAUU nonamer. The list of mRNAs bearing such elements has since then greatly expanded, revealing that ARE-containing mRNAs encode a wide repertoire of functionally diverse proteins [2]. AREs were at first shown to mediate RNA instability when inserted into the 3′UTR of reporter genes [3]. Based on the number