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Tristetraprolin Recruits Functional mRNA Decay Complexes to ARE Sequences

Heidi H. Hau,[1] Richard J. Walsh,[1] Rachel L. Ogilvie,[1] Darlisha A. Williams,[1] Cavan S. Reilly,[3] and Paul R. Bohjanen[1,2]*

[1]Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455
[2]Department of Medicine, Division of Infectious Diseases and International Medicine, Medical School, University of Minnesota, Minneapolis, Minnesota 55455
[3]Division of Biostatistics, School of Public Health, University of Minnesota, Minneapolis, Minnesota 55455

Abstract AU-rich elements (AREs) in the 3′ untranslated region (UTR) of numerous mammalian transcripts function as instability elements that promote rapid mRNA degradation. Tristetraprolin (TTP) is an ARE-binding protein that promotes rapid mRNA decay through mechanisms that are poorly understood. A 31 nucleotide ARE sequences from the TNF-alpha 3′ UTR promoted TTP-dependent mRNA decay when it was inserted into the 3′ UTR of a beta-globin reporter transcript, indicating that this short sequence was sufficient for TTP function. We used a gel shift assay to identify a TTP-containing complex in cytoplasmic extracts from TTP-transfected HeLa cells that bound specifically to short ARE sequences. This TTP-containing complex also contained the 5′–3′ exonuclease Xrn1 and the exosome component PM-scl75 because it was super-shifted with anti-Xrn1 or anti-PMscl75 antibodies. RNA affinity purification verified that these proteins associated specifically with ARE sequences in a TTP-dependent manner. Using a competition binding assay, we found that the TTP-containing complex bound with high affinity to short ARE sequences from GM-CSF, IL-3, TNF-alpha, IL-2, and c-fos, but did not bind to a U-rich sequence from c-myc, a 22 nucleotide poly U sequence or a mutated GM-CSF control sequence. High affinity binding by the TTP-containing complex correlated with TTP-dependent deadenylation and decay of capped, polyadenylated transcripts in a cell-free mRNA decay assay, suggesting that the TTP-containing complex was functional. These data support a model whereby TTP functions to enhance mRNA decay by recruiting components of the cellular mRNA decay machinery to the transcript. J. Cell. Biochem. 100: 1477–1492, 2007.

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The cellular response to physiological and environmental cues requires a tightly controlled, coordinated regulation of gene expression at multiple levels. Although transcription is a major control point, post-transcriptional mechanisms also play pivotal roles in regulating gene expression. An important level of post-transcriptional control occurs at the level of mRNA stability. The decay of a variety of short-lived transcripts is mediated by *cis*-acting sequences known as AU-rich elements (AREs)[1] that are found in the 3′ untranslated region (UTR). The best characterized AREs are found in cytokine transcripts and other early response gene transcripts, but AREs or ARE-like sequences are found in a large and diverse repertoire of cellular transcripts that are regulated in response to environmental stimuli [Bakheet et al., 2001, 2003, 2006; Raghavan et al., 2002, 2004; Vlasova et al., 2005]. Although AREs function as instability elements [Caput et al., 1986; Shaw and Kamen, 1986], they also regulate mRNA localization [Veyrune et al., 1996] and translation [Kruys et al., 1989; Han et al., 1990]. The mechanisms by which

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*Correspondence to: Paul R. Bohjanen, Department of Microbiology, University of Minnesota, 420 Delaware Street SE, MMC 196, Minneapolis, MN 55455.
E-mail: bohja001@umn.edu

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AREs mediate instability are poorly understood, but it is becoming clear that interactions with ARE-binding proteins play an important role.

Several ARE-binding proteins have been identified that influence mRNA turnover, but exact mechanisms by which this occurs are not fully understood. Members of the embryonic lethal abnormal vision family of RNA binding proteins, including HuR (also called HuA) and HuB, bind to AREs and mediate the stabilization of ARE-containing transcripts [Jain et al., 1997; Fan and Steitz, 1998; Levy et al., 1998; Peng et al., 1998; Ford et al., 1999]. ARE and poly-U-binding degradation factor 1, which consists of 37, 40, 42, and 45 kDa protein isoforms [Wagner et al., 1998], plays a role in both destabilization [Sarkar et al., 2003] and heat shock-induced stabilization [Laroia et al., 1999] of ARE-containing transcripts. K homology splicing regulatory protein is an ARE-binding protein that can associate directly with both poly A ribonuclease (PARN) and the exosome, a multi protein complex consisting of 3′–5′ exoribonucleases and helicases, and may promote ARE-mediated mRNA decay through recruitment of these components of the mRNA decay machinery [Chen et al., 2001; Gherzi et al., 2004].

Three members of the TPA-induced sequence 11 (TIS11) protein family, including Tristetraprolin (TTP/ TIS11), butyrate response factor-1 (BRF-1/ TIS11b) and butyrate response factor-2 (BRF-2/ TIS11d), can promote ARE-directed deadenylation and mRNA decay [Lai et al., 2000]. TTP has been the most intensely studied of the three proteins, and has been shown in a knock-out mouse model to promote the decay of tumor necrosis factor-alpha (TNF-alpha) [Carballo et al., 1998; Lai et al., 1999], granulocyte macrophage-colony stimulating factor (GM-CSF) [Carballo et al., 2000], and interleukin-2 (IL-2) [Ogilvie et al., 2005] transcripts. Overexpression of TTP promoted the decay of exogenously expressed reporter transcripts that contained AU-rich sequences from TNF-alpha [Carballo et al., 1998; Lai et al., 1999; Chen et al., 2001; Blackshear, 2002], IL-2 [Ogilvie et al., 2005], GM-CSF [Carballo et al., 1998], interleukin-3 (IL-3) [Carballo et al., 1998; Stoecklin et al., 2000], and c-jun [Briata et al., 2003]. Perhaps, TTP and other TIS11 family members promote ARE-mediated mRNA decay through an interaction with components of the cellular mRNA decay machinery. All three TIS11 family members stimulated PARN activity in a cell-free system [Lai et al., 2003]. Also, TTP and BRF-1 have been shown to co-immunoprecipitate with components of the cellular mRNA decay machinery, including the decapping enzyme Dcp1, the 5′–3′ exonuclease Xrn1, subunits of the exosome, and the deadenylase Ccr4 [Blackshear, 2002; Lykke-Andersen and Wagner, 2005]. TTP has also been shown to interact with Argonaute/eiF2C family members, components of micro RNA (miRNA) processing and function [Jing et al., 2005], and MiR16, a miRNA bearing a complementary sequence to a portion of the TNF-alpha ARE, was found to be required for TTP-dependent ARE-mediated turnover of the TNF-alpha mRNA transcript [Jing et al., 2005]. Although it is not clear if TTP can associate directly with Argonaute/eiF2C or miR16, it appears both are required for TTP-mediated decay of the TNF-alpha transcript [Jing et al., 2005].

Structure-function analyses of the TTP and BRF-1 proteins have shown that the CCCH zinc fingers are responsible for binding to the ARE [Lai et al., 1999], while the N-terminal and C-terminal domains serve as activation domains for mRNA decay [Lykke-Andersen and Wagner, 2005]. TTP must bind to the ARE in order to promote deadenylation and subsequent decay of ARE-containing transcripts. TNF-alpha reporter transcripts that contain a mutated ARE were not degraded in a TTP-dependent manner [Lai et al., 1999]. Also, TTP mutants made defective in binding by mutation of cysteine to arginine in either of the zinc fingers, remained polyadenylated and showed little or no decay [Lai et al., 1999], suggesting that both CCCH zinc finger domains are required to bind to AREs and promote deadenylation and decay [Lai et al., 1999]. A synthetic 73 amino acid peptide containing two tandem TTP CCCH zinc fingers bound to a TNF-alpha ARE sequence with high affinity [Blackshear et al., 2003; Brewer et al., 2004], whereas a synthetic 36-amino acid peptide that contained only the first CCCH zinc finger bound to a UUUAUUU sequence but with much lower affinity [Michel et al., 2003]. The N-terminal and C-terminal domains of TTP each serve as activation domains for mRNA decay and can promote mRNA decay in the absence of an ARE if tethered to the RNA through a heterologous binding site [Lykke-Andersen and Wagner, 2005]. The N-terminal

domain of TTP interacts with components of the mRNA decay machinery including the decapping enzyme Dcp1, the 5′–3′ exonuclease Xrn1, and the exosome [Chen et al., 2001; Lykke-Andersen and Wagner, 2005]. The C-terminal domain of TTP also functions to promote activation of decay through an unidentified mechanism [Lykke-Andersen and Wagner, 2005].

TTP has been shown to bind to a variety of AU-rich sequences found in the 3′ UTR of short-lived transcripts, including sequences from TNF-alpha [Raghavan et al., 2001; Blackshear et al., 2003; Brewer et al., 2004; Cao, 2004], GM-CSF [Raghavan et al., 2001], IL-2 [Raghavan et al., 2001; Ogilvie et al., 2005], IL-3 [Raghavan et al., 2001], and c-fos [Raghavan et al., 2001; Worthington et al., 2002]. Many of these TTP-binding sites are components of functional AREs that have been shown to mediate mRNA decay [Chen and Shyu, 1995]. Functional AREs have been categorized based on their sequence characteristics and decay kinetics [Chen and Shyu, 1995; Xu et al., 1997]. Class I AREs contain dispersed copies of AUUUA in the context of other U-rich sequences, class II AREs contain tandemly reiterated copies of the AUUUA motif, and class III AREs contain U-rich sequences in the absence of the AUUUA motif. The known TTP-binding sites found in the 3′ UTR of cytokine transcripts resemble class II AREs, whereas the TTP-binding site found in the c-fos 3′ UTR is part of a functional class I ARE [Chen and Shyu, 1995; Raghavan et al., 2001]. Utilizing RNA SELEX, Worthington et al. [2002] demonstrated that TTP has a preference for binding to the 9 bp consensus sequence UUAUUUAUU. Brewer et al. [2004] showed that a synthesized 73 amino acid TTP peptide bound with high affinity to AU-rich sequences containing two adenylates preceding 3-6 uridylates followed by two more adenylates (AAUUUAA, AAUUUUAA, AAUUUUUAA, AA UUUUUUUAA). In that study, high affinity binding was also shown for AUUUA or AUUUUA sequences from the TNF-alpha transcript [Brewer et al., 2004]. Quantitative information about the affinity of TTP is known for only a small number of functional ARE sequences [Blackshear et al., 2003; Brewer et al., 2004; Cao, 2004], and no comprehensive studies have been performed to determine if high affinity TTP binding correlates with mRNA decay function.

We have shown that very short (31–32 nucleotide) AU-rich sequences from the IL-2 [Ogilvie et al., 2005] and TNF-alpha transcripts mediate TTP-dependent mRNA decay when they are inserted into the 3′ UTR of a beta-globin reporter transcript, indicating that these short ARE sequences are sufficient for TTP function. The findings that TTP can associate with components of the cellular mRNA decay machinery has led to the hypothesis that TTP does not bind to AU-rich sequences as a single isolated protein but as a complex of proteins [Jing et al., 2005; Lykke-Andersen and Wagner, 2005]. We therefore conducted RNA-binding experiments in cellular cytoplasmic extracts that contained TTP as well as other proteins involved in mRNA decay in order to better characterize the binding of TTP to functional ARE sequences. We found that TTP binds to ARE sequences as a complex that contains the 3′–5′ exonuclease Xrn1 and the exosome component PM-scl75. In this system, we found that the TTP-containing complex bound with high affinity to short AU-rich sequences from TNF-alpha, GM-CSF, IL-2, and c-fos but not to a U-rich sequence from c-myc or a mutated GM-CSF sequence. High affinity binding by the TTP-containing complex correlated with TTP-dependent deadenylation and decay of capped, polyadenylated transcripts that contained these sequences, suggesting that the TTP-containing complex was functional. These data support a model whereby TTP functions to enhance mRNA decay by recruiting components of the cellular mRNA decay machinery to the transcript.

MATERIALS AND METHODS

mRNA Decay Assay in Tet-Off HeLa Cells

Tet-off HeLa cells (BD Clontech) were grown in Dulbeco's Modified Eagle Media (Invitrogen Life Technologies) with 10% Tet-approved fetal bovine serum (BD Biosciences), 4 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. These cells (2.0×10^5 cells) were transfected using TransIt LT1 reagent (Mirus) with 90 ng of the pCMV.TTP.Tag plasmid [Carballo et al., 1998], which encodes the full-length human TTP cDNA, linked to a hemagglutinin (HA) tag or the pcDNA-3 plasmid (Invitrogen Life Technologies) as a mock control. These cells were also transfected with 270 ng of the pTracer-EF/V5-His/lacZ construct

(Invitrogen Life Technologies) which produces green fluorescent protein (GFP) to control for transfection efficiency, and with 330 ng of a Tet-responsive reporter construct encoding the rabbit beta-globin transcript that contained a TNF-alpha ARE sequence (pTetBBB/TNF) or a mutated ARE sequence (pTetBBB/M8) inserted into its 3′ UTR. These plasmids were derived from pTetBBB [Chen and Shyu, 1994; Chen et al., 1994] by inserting the sequence AUUU AUUAUUUAUUUAUUAUUUAUUUAUUUA (pTetBBB/TNF) or ACUCACUCACUCACUC ACUCA (pTetBBB/M8) into the rabbit beta-globin 3′ UTR at a unique Bgl II restriction site. Doxycycline (20 μg/ml) was added 48 h after the transfection to stop transcription from the pTet constructs, and total RNA was isolated after 0, 30, 60, or 90 min as described previously [Ogilvie et al., 2005]. Total RNA (5 μg from each sample) was separated by electrophoresis on 1.2% glyoxal agarose gels using the NorthernMax-Gly system (Ambion). The RNA was blotted onto Brightstar-Plus membranes (Ambion) and immobilized onto the membrane by irradiation with 254 nm UV light using a Stratalinker cross-linking apparatus (Stratagene). Blots were hybridized overnight with radiolabeled rabbit beta-globin or GFP-DNA probes as described previously [Ogilvie et al., 2005]. The blots were washed, and signal intensities were quantified using a PhosphorImager (Molecular Dynamics). The hybridization intensity of each transcript was normalized to the hybridization intensity of the GFP transcript and the normalized values were used to calculate transcript half-lives.

Preparation of Cytoplasmic Extracts and S100 Extracts

HeLa cells were grown in monolayers in Iscove's Modified Dulbecco's Medium (Invitrogen Life Technologies) supplemented with 10% bovine calf serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Approximately 1.0×10^7 HeLa cells were transfected using TransIT LT1 reagent (Mirus) with 10–50 μg of pCMV.TTP.Tag plasmid [Carballo et al., 2001] or the pcDNA-3 plasmid (Invitrogen Life Technologies) as a mock control. After 48 h, the cells were harvested. Cytoplasmic extracts were prepared by lysis of cells in a buffer containing 0.2% Nonidet P-40, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, 8 ng/ml aprotinin, 2 ng/ml leupeptin, and 0.5 mM PMSF. Nuclei were removed by centrifugation at 14,000 rpm for 2 min in an Eppendorf microcentrifuge. S100 extracts were prepared as previously described [Fritz et al., 2000]. Cytoplasmic extracts and S100 extracts were immediately frozen on dry ice and stored at −80°C. The protein concentration of extracts was determined by a colorimetric assay using a commercially available reagent (Bio-Rad) according to the manufacturer's instructions.

RNA Oligonucleotide Probes and Competitors

RNA oligonucleotides used as probes or competitors were purchased commercially (Dharmacon Research). Table I shows the sequence of the RNA oligonucleotides used. Radioactive RNA probes were end-labeled with 4,500 Ci/mmol (gamma-^{32}P) ATP (ICN Biochemicals) using T4 polynucleotide kinase (Promega). Specific activities were approximately 1.5×10^3 cpm/fmol.

Gel Shift and Super-Shift Assays

Gel shift assays were conducted by incubating cytoplasmic extracts with a ^{32}P-labeled GM-CSF RNA probe at room temperature for 20–30 min in a buffer containing 0.2% Nonidet P-40, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol in the presence of 5 mg/ml heparan sulfate (Sigma) and 500 pmol/ml of poly(U) (M1) RNA. Each

TABLE I. Apparent Affinities of the TTP-Containing Complex for RNA Sequences

RNA	Sequence	Apparent affinity	Standard error
GM-CSF	AUUUAUUUAUUUAUUUAUUUA	21 nM	35 nM
mGM-CSF (M8)	ACUCACUCACUCACUCACUCA	>1 μM	ND
Poly U (M1)	UUUUUUUUUUUUUUUUUUUUU	>1 μM	ND
c-myc	UUACCAUCUUUUUUUUUCUUUA	>1 μM	ND
c-fos	UAUUUAUAUUUUUAUUUUAUUU	34 nM	16 nM
TNF-alpha	AUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA	2 nM	2 nM
IL-2	UAUUUAUUUAAAUAUUUAAAUUUUAUAUUUAU	44 nM	46 nM
IL-3	UAUUUAUUUAUGUAUUUAUGUAUUUAUUUAUUUAU	4 nM	4 nM

reaction contained 10–25 μg of cytoplasmic protein, 25–35 fmol of radiolabeled GM-CSF RNA probe, and increasing amounts of the unlabeled competitor RNA oligonucleotides shown in Table I. For super-shift assays, specific antibodies or control antibodies were added to the reaction mixtures. The anti-TTP (N-18) antibody used was raised against the amino terminus of human TTP and an anti-actin (C-11) antibody was used as a control; both were affinity purified goat polyclonal antibodies that were purchased commercially (Santa Cruz Biotechnologies). The anti-hemagglutinin (HA) (Y-11) antibody and the anti-Akt1/2 (H-136) control antibodies were both affinity-purified rabbit polyclonal antibodies that were purchased commercially (Santa Cruz Biotechnologies). Rabbit anti-PM-Scl75 and anti-Xrn1 polyclonal antisera were generous gifts from Dr. Jeff Wilusz (Colorado State University, Fort Collins, CO) and Dr. Jens Lykke-Andersen (University of Colorado, Boulder, CO), respectively. After the addition of antibodies, reaction mixtures were incubated on ice for 2–20 min and were then separated by electrophoresis under non-denaturing conditions on 5%-polyacrylamide gels using 1% Tris-Glycine EDTA running buffer. The gels were dried and analyzed on a phosphorimager (Molecular Dynamics). Where indicated, the binding intensity of the RNA-protein complexes and free RNA were quantified using Image-Quant version 5.2 software (Molecular Dynamics). GraphPad Prism version 4.03 for Windows, (GraphPad Software, San Diego, CA) was used to graph binding data and calculate apparent affinities and standard errors using a homologous competition with depletion one-site binding model or a heterologous competition with depletion one-site binding model.

RNA Affinity Purification of Protein Complexes

Cytoplasmic extracts were incubated at room temperature for 15 min with a 3′ biotinylated GM-CSF ARE (AUUUAUUUAUUUAUUUA UUUA) RNA or mutated ARE (ACUCACUCACUCACUCACUCA) RNA (M8) which were purchased commercially (Dharmacon). Streptavidin-coated agarose beads (Pierce) or magnetic beads (Dynal) were washed with a bead wash buffer containing 10 mM HEPES (pH 7.9), 40 mM KCl, 3 mM $MgCl_2$, 10% glycerol, and 1 mM DTT. These beads were added to the binding mixtures and were incubated at room temperature with rotation for 40 min. The beads were washed with bead wash buffer and material on the beads was either eluted with a buffer containing 8 M Urea/2% CHAPS or else beads were directly loaded onto SDS-polyacrylamide gels.

Western Blotting

Protein samples separated by SDS–PAGE were electro-blotted onto Immobilon P membranes (Millipore). Western blots were probed with an affinity purified rabbit polyclonal anti-HA (Y11) antibody purchased commercially (Santa Cruz Biotechnologies), a rabbit polyclonal anti-Xrn1 antibody (a generous gift from Dr. Jens Lykke-Anderson), a rabbit polyclonal anti-PMScl75 antibody (a generous gift from Dr. Jeff Wilusz), a goat polyclonal anti-actin antibody (Santa Cruz Biotechnologies), or a mouse monoclonal anti-poly A binding protein antibody (Ab 6125) purchased commercially (Abcam, Inc.). Antibody binding was detected by secondary anti-rabbit or anti-mouse antibodies conjugated to horse radish peroxidase (Amersham Biosciences) and blots were visualized by addition of SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure to Hyperfilm (Amersham Biosciences).

Preparation of Capped Polyadenylated RNA Substrates for Cell-Free Decay

DNA template sequences were cloned into the pGEM EasyT (Promega) vector system according to manufacturer's instructions. Each template sequence was prepared from four separate ssDNA oligos that were annealed and amplified by PCR prior to cloning. The template sequences were designed to produce polyadenylated transcripts that contained the GM-CSF, IL-2, TNF-alpha, c-myc, and M8 sequences shown in Table I. An NsiI site was put immediately downstream of a 62 bp poly A tail such that after restriction enzyme digestion of the plasmid, there was no remaining sequence after the last adenine. Capped, ^{32}P-internally labeled RNA substrate molecules were synthesized using T7 RNA polymerase from Nsi I linearized plasmids as previously described [Fritz et al., 2000]. The resulting transcripts had the following sequence: 5′ GGGCGAA UUGGGCCCGACGUCGCAUGCUCCCGGCC GCCAUGGCCGCGGGAUUGCGCGAUACGA AUUCGAGCUCGGUAC-insert-GATTCAAGC

TTCTGGCA$_{(62)}$3′ where "insert" represents the GM-CSF, IL-2, TNF-alpha, c-myc, and M8 sequences shown in Table I.

Cell-Free mRNA Decay Assay

RNA decay assays were conducted according to Fritz et al. [2000] with minor modifications. S100 extracts containing 25–50 μg of cytoplasmic protein were mixed with 25–100 fmol of radiolabeled RNA substrate in the presence of 15 mM ATP, 200 mM phosphocreatine, 10% polyvinyl alcohol, and 250 ng/μl poly A RNA in a total volume of 26 μl. Reactions were stopped after 0, 0.5, 2, or 4 h and purified RNA was separated by electrophoresis on 10% polyacrylamide-urea gels. Gels were dried and bands were visualized using a PhosphorImager (Molecular Dynamics).

RESULTS

TTP Promotes the Decay of Transcripts That Contain Short ARE Sequences

TTP regulates the decay of a variety of cytokine transcripts, including TNF-alpha, GM-CSF, and IL-2 [Carballo et al., 1998, 2000; Lai et al., 1999; Carrick et al., 2004; Ogilvie et al., 2005], presumably by binding to the AREs found in these sequences and regulating the mRNA decay machinery. We used a transient transfection system in HeLa cells to determine if TTP regulates the decay of a reporter transcript that contains a short AU-rich sequence that could function as a TTP binding site. HeLa Tet-off cells were transiently transfected with altered versions of the tetracycline-repressible reporter plasmid pTetBBB [Chen and Shyu, 1994; Chen et al., 1994] that expresses the rabbit beta-globin transcript. The wild-type rabbit beta-globin sequence was altered such that a 31 nucleotide ARE sequence from TNF-alpha or a mutated ARE sequence (M8) was inserted into the 3′ UTR to create the pTetBBB/TNF and pTetBBB/M8 plasmids, respectively. Each of these plasmids was transfected into HeLa cells along with a GFP expression plasmid to control for transfection efficiency and either a TTP expression plasmid or a mock control plasmid. Doxycycline was added to the cells 48 h post-transfection to stop transcription from the beta-globin reporter plasmids, and total cellular RNA was harvested after 0, 30, 60, and 90 min. Beta-globin and GFP mRNA levels were assessed by Northern blot (Fig. 1A), and beta-globin mRNA expression was normalized to GFP mRNA expression. This data was graphed for each time point and mRNA decay rates were calculated based on a one-phase model of exponential decay (Fig. 1B). The BBB/M8 transcript was very stable in

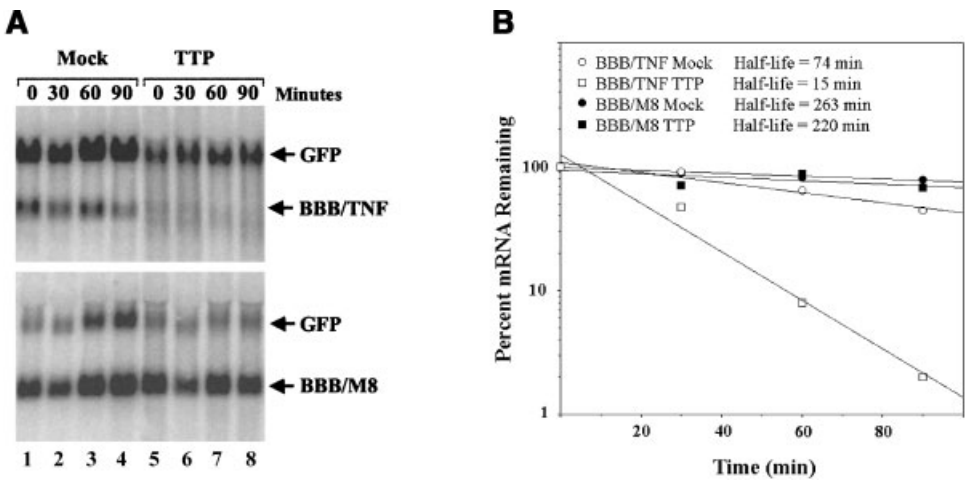

Fig. 1. Overexpression of TTP leads to enhanced decay of transcripts that contain short ARE sequences. **A**: HeLa Tet-off cells were transfected with pTetBBB/TNF or pTetBBB/M8 plasmids in the presence of the pCMV.TTP.Tag plasmid (TTP) or a pcDNA3 control plasmid (mock). The pTracer GFP expression construct was cotransfected in each group as a control for transfection efficiency. After 48 h, doxycycline was added to the culture medium to stop transcription from the pTetBBB plasmids and total RNA was isolated after 0, 30, 60, or 90 min. For each sample, mRNA expression was evaluated by Northern blot using ^{32}P-labeled rabbit beta-globin (BBB) and GFP probes. **B**: The Northern blots shown in **panel A** were quantified using a phosphorimager, and the hybridization intensity of the full-length beta-globin (BBB) mRNA bands were normalized to GFP mRNA bands. For each sample, the normalized value for beta-globin expression at time 0 was set to 100%. Transcript half-lives were calculated based on a first order decay model.

mock-transfected cells with a half-life of 263 min. In contrast, the BBB/TNF transcript was much less stable in mock-transfected cells with a half-life of only 74 min. This result suggests that the 31 nucleotide ARE sequence in the BBB/TNF transcript is sufficient to mediate mRNA decay in HeLa cells. Overexpression of TTP further destabilized the pTetBBB/TNF transcript (half life of 15 min), suggesting that TTP functions to promote the decay of a transcript that contains a very short (31 nucleotide) ARE. The more rapid decay of the BBB/TNF transcript in TTP-transfected cells compared to mock-transfected cells explains the lower steady state level of the transcript at the zero time point (compare BBB/TNF transcript levels in lanes 1 and 5). Overexpression of TTP had little effect on the decay of the BBB/M8 transcript, suggesting that the function of TTP is ARE-specific. Overall, our results suggest that the 31 nucleotide TNF-alpha ARE is sufficient for TTP-mediated mRNA decay. These results are consistent with our previous observation that a 32 nucleotide ARE sequence from the IL-2 3′UTR was also sufficient to allow TTP-dependent decay of the same beta-globin reporter [Ogilvie et al., 2005].

TTP Recruits Components of the mRNA Decay Machinery to Short ARE Sequences

TTP has been shown to facilitate the deadenylation [Lai et al., 1999] and subsequent degradation [Chen et al., 2001; Mukherjee et al., 2002] of ARE-containing transcripts, but the mechanisms by which TTP functions are still not clear. An association between TTP and the mammalian exosome has been reported [Chen et al., 2001]. In experiments conducted with TTP-depleted S100 extracts from Jurkat cells, 3′–5′ exosomal-mediated decay was abolished for transcripts that contained the 3′ UTR from IL-2 or AREs from TNF-alpha and c-fos [Chen et al., 2001]. After addition of recombinant TTP, decay was restored suggesting that TTP was needed for exosomal activity [Chen et al., 2001]. Moreover, the N-terminal domain of TTP was found to associate in an RNA-independent manner with enzymes involved in mRNA deadenylation (Ccr4), decapping (Dcp1a), 3′–5′ exosomal-mediated decay (PM-Scl75) and 5′–3′ decay (Xrn1) [Lykke-Andersen and Wagner, 2005]. Therefore, we hypothesized that TTP may bind to RNA as a part of a multiprotein complex that also contains components of the mRNA decay machinery. To test this hypothesis, we performed gel shift experiments to determine if TTP-containing complexes that form on a short AU-rich sequence could be super-shifted using antibodies directed against components of the mRNA decay machinery.

Gel shift assays were performed using cytoplasmic extracts from HeLa cells that were mock transfected or were transfected with the pCMV.TTP.Tag expression plasmid and a short radiolabeled ribo-oligonucleotide probe that consisted of 22 nucleotides from the GM-CSF ARE (see Table I). Cytoplasmic extracts from mock-transfected HeLa cells expressed little endogenous RNA-binding activity that bound to the GM-CSF AU-rich probe (Fig. 2, lane 2). Also, endogenous TTP expression in cytoplasmic extracts from mock-transfected HeLa cells was undetectable by Western blot but was abundant in extracts from TTP-transfected cells (data not shown). When a gel shift assay was performed using cytoplasmic extracts from TTP-transfected HeLa cells, a single dominant shifted complex was observed (lane 3). This dominant complex contained TTP because it was completely and specifically super-shifted by an anti-HA antibody that recognizes the HA-tag on the overexpressed TTP (lane 4) or by an anti-TTP antibody (lane 6) but was not super-shifted by control antibodies (lanes 5 and 7).

Experiments were performed to determine if antibodies against mRNA decay components could super-shift the TTP-containing complex (Fig. 3). As was also seen in Figure 2, a predominant shifted complex was observed when an extract from TTP-transfected cells was used (lane 2), and this complex was completely super-shifted with an anti-HA antibody that recognized the HA-tag on the transfected TTP (lane 6) but not with a control antibody (lane 4). As expected, neither the control antibody nor the anti-HA antibody caused a super-shifted complex in extracts from mock-transfected cells (lanes 3 and 5, respectively). Interestingly, an anti-PM-scl75 antibody and an anti-Xrn1 antibody caused the TTP-containing complex to be super-shifted suggesting that PM-scl75 and Xrn1 are components of the TTP-containing complex. The anti-PM-scl75 antibody and the anti-hXrn-1 antibody did not cause super-shifted complexes to form when extracts from mock-transfected cells were used (lanes 7 and 9), suggesting that the association of PM-scl75 and Xrn1 with the RNA

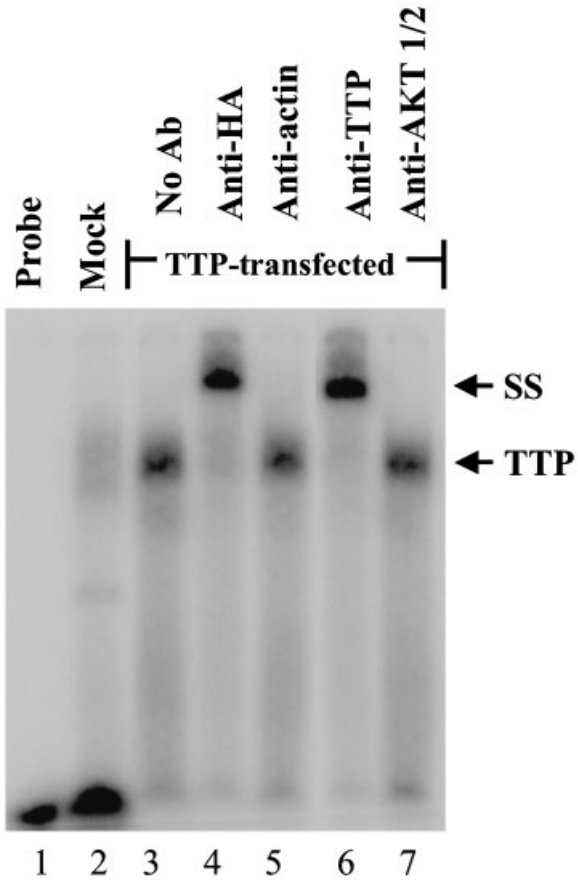

Fig. 2. TTP-containing complexes associate with short ARE sequences. HeLa cells were transiently transfected with 50 μg of pcDNA-3 (mock) or pCMV.TTP.HA-Tag (TTP) plasmids, and cytoplasmic extracts were harvested 48 h later. An RNA/protein gel shift assay was performed by mixing cytoplasmic extracts containing 10 μg of total protein from mock (**lane 2**) or TTP-transfected HeLa cells (**lanes 3–7**) with 35 fmol of a ^{32}P end-labeled GM-CSF RNA probe. The following antibodies were added to the indicated reaction mixtures: goat polyclonal anti-HA antibody (lane 4), goat polyclonal anti-actin control antibody (lane 5), rabbit polyclonal anti-TTP antibody (lane 6), or rabbit polyclonal anti-AKT1/2 antibody (lane 7). The binding reactions were then separated by electrophoresis on a 5% polyacrylamide gel under non-denaturing conditions and bands were visualized using a phosphorimager. The position of the TTP-containing band (TTP) and super-shifted bands (SS) are indicated. The position of migration of the probe alone is seen in **lane 1**.

was dependent on the presence of transfected TTP. Identical results were obtained when S100 extracts were used to perform the experiments (Fig. 3, right). Since TTP has been shown to associate with PM-scl75 and Xrn1 in an RNA-independent manner [Lykke-Andersen and Wagner, 2005], the simplest interpretation of these results is that TTP functioned to recruit PM-scl75 and Xrn1 to the GM-CSF ARE RNA probe.

An RNA affinity purification approach was used to confirm that TTP recruited PM-scl75 and Xrn1 to an AU-rich RNA. In this approach, a 22 nucleotide biotinylated RNA containing the GM-CSF ARE sequence or a mutated ARE sequence (M8) was incubated with mock or TTP-transfected HeLa cytoplasmic extracts to allow TTP-containing complexes to form on the RNA. The reaction mixtures were then incubated with streptavidin beads, the beads were washed, and proteins associated with the beads, washes or supernatants were analyzed by Western blot using an anti-HA antibody to detected HA-tagged TTP or an anti-actin antibody as a control (Fig. 4A). When the biotinylated GM-CSF ARE ribo-oligonucleotide was used, TTP was specifically recruited to the beads (lane 6) and depleted from the supernatant (lane 2). In contrast, TTP remained in the supernatant (lane 3) and did not associate with the beads (lane 7) when the M8 mutant ARE ribo-oligonucleotide was used. Xrn1 and PM-scl75 were co-purified with TTP using this RNA affinity purification approach when the biotinylated GM-CSF ARE ribo-oligonucleotide was used but not when the M8 ribo-oligonucleotide was used (Fig. 4B). No purification of Xrn1 or PM-scl75 occurred when cytoplasmic extracts from mock-transfected HeLa cells were used, confirming that Xrn1 and PM-Scl75 were recruited to the GM-CSF ARE sequence in a TTP-dependent fashion. Taken together, our affinity purification and super-shift experiments show for the first time that Xrn1 and PM-scl75, proteins known to mediate mRNA turnover, are recruited to AU-rich sequences in a TTP-dependent manner.

TTP-Containing Complexes Bind With High Affinity to Short AU-Rich Sequences From Cytokine and Proto-Oncogene Transcripts

Our research group and others have shown that TTP binds to AU-rich sequences found in the 3′ UTR of a variety of cytokine and proto-oncogene transcripts [Raghavan et al., 2001; Worthington et al., 2002; Blackshear et al., 2003; Brewer et al., 2004; Cao, 2004; Ogilvie et al., 2005]. We hypothesized that TTP binds to specific high affinity sites within AREs and targets the bound transcripts for rapid decay as part of a down regulatory mechanism to turn off expression of early response genes. Based on our

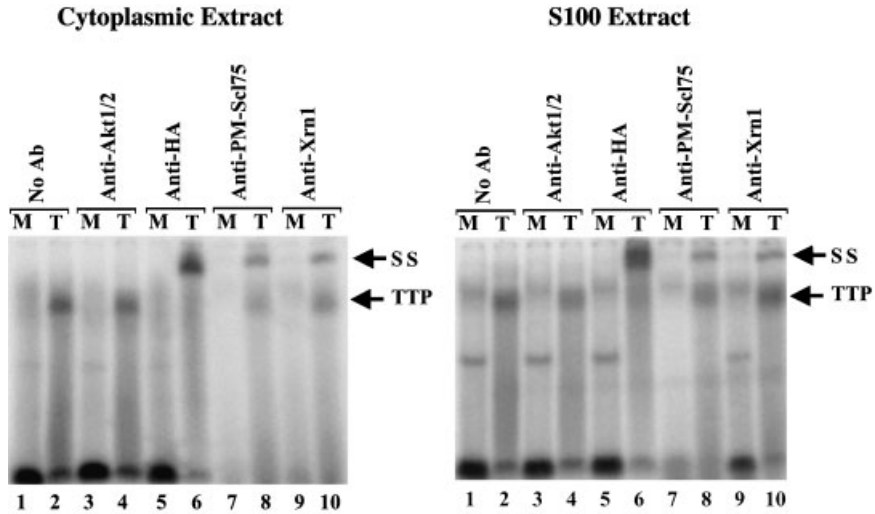

Fig. 3. TTP recruits PM-Scl75 and Xrn1 to ARE-containing RNA. HeLa cells were transiently transfected with 10 μg of the pcDNA-3 plasmid (M) or 10 μg of the pCMV.TTP.HA-Tag plasmid (T), and cytoplasmic extracts (**left panel**) or S100 extracts (**right panel**) were prepared 48 h later. RNA protein gel shift assays were performed by mixing extracts containing 25 μg of total protein with 25 fmol of a ^{32}P end-labeled GM-CSF RNA probe. The following antibodies were added to the indicated binding reactions: no antibody (**lanes 1** and **2**), anti-AKT1/2 control antibody (**lanes 3** and **4**), anti-HA antibody (**lanes 5** and **6**), anti-PM-Scl75 antibody (**lanes 7** and **8**), and anti-Xrn1 antibody (**lanes 9** and **10**). The binding reactions were then separated by electrophoresis on a 5% polyacrylamide gel under non-denaturing conditions and visualized using a phosphorimager. The positions of the TTP-containing complex (TTP) and super-shifted complexes (SS) are indicated.

results, it appears that TTP does not function alone but binds to AREs as part of a larger complex of proteins. We used a gel shift assay to quantitatively assess the binding by the TTP-containing complex present in crude HeLa cytoplasmic extracts to AU-rich sequences found in the 3′ UTRs of various cytokine or proto-oncogene transcripts. By using crude

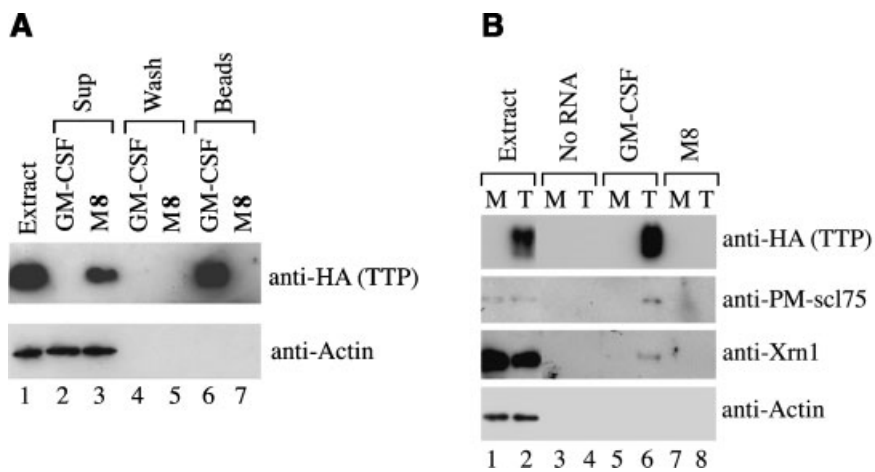

Fig. 4. Purification of TTP-containing complexes using RNA affinity chromatography. **A**: A biotinylated GM-CSF ARE ribo-oligonucleotide (GM-CSF) or a biotinylated M8 mutated GM-CSF ARE ribo-oligonucleotide (M8) were incubated with TTP-containing HeLa cell cytoplasmic extracts. Streptavidin-coated agarose beads were added, and the beads were washed. Extract alone (**lane 1**) or material from the supernatants (Sup, **lanes 2** and **3**) washes (wash, **lanes 4** and **5**) or beads (**lanes 6** and **7**) were analyzed by Western blot with anti-HA and anti-actin antibodies. **B**: Cytoplasmic extracts prepared from mock-transfected (M) or TTP-transfected (T) HeLa cells were incubated with a biotinylated GM-CSF ARE ribo-oligonucleotide (GM-CSF), a biotinylated M8 mutated GM-CSF ARE ribo-oligonucleotide (M8), or no additional RNA (No RNA). Streptavidin-coated magnetic beads were added, and the beads were washed. Extract alone (lanes 1 and 2) or material from the beads (lanes 3–7) were analyzed by Western blot with anti-HA, anti-PM-scl75, anti-Xrn1, and anti-actin antibodies.

cytoplasmic extracts rather than purified recombinant TTP protein, we could assess the binding of TTP-containing complexes in a manner that may be more physiologically relevant.

We chose to measure the affinities of the binding by the TTP-containing complex to short AU-rich sequences from the AREs of cytokine and proto-oncogene transcripts that exhibit very rapid decay (Table I). The GM-CSF, IL-3, TNF-alpha, and IL-2 sequences were derived from class II AREs and all contain reiterated AUUUA pentamer sequences. The c-fos and c-myc sequences were derived from class I AREs; the c-fos sequence contains two non-reiterated AUUUA pentamer sequences but the c-myc sequence was derived from a U-rich portion of the ARE that does not contain any AUUUA pentamers. This U-rich tract in the c-myc sequence is a known binding site for the ARE-binding protein HuR [Raghavan et al., 2001]. The M1 sequence is a 22 nucleotide poly U tract that binds to the HuR protein [Raghavan et al., 2001], and the M8 sequence is a mutated version of the GM-CSF AU-rich sequence. TTP binding sequences have been shown to contain the AU-rich nonamer sequence UUAUUUAUU [Worthington et al., 2002] or to contain AUUUA or AUUUUA sequences in the context of other AU-rich or U-rich sequences [Brewer et al., 2004]. The AU-rich sequences in Table I provide a variety of these potential binding sites that were tested in a competition binding assay. Gel shift experiments were performed using cytoplasmic extracts from TTP-transfected HeLa cells and the 22 nucleotide radiolabeled GM-CSF AU-rich probe in the presence of increasing amounts of unlabeled competitor RNA (Fig. 5). Each sequence shown in Table I was used as a competitor in three independent experiments. Representative gels from one of three experiments are shown for each competitor sequence and to the right of each gel is a graph showing combined quantitative competition data from the three experiments. Figure 6 shows the same data represented together on one graph to allow easier comparisons. Graph Pad Prism software was used to generate the competition curves and to calculate apparent affinities (K_d). High affinity binding was noted for the interaction between the TTP-containing complex and the GM-CSF, IL-3, TNF-alpha, IL-2, and c-fos AU-rich sequences with apparent Kd values ranging from 2 to 44 nM. The variability of the assay, however, did not allow us to make comparative conclusions about the relative binding affinities for these sequences. These affinity measurements performed in crude extracts are similar to previously reported interactions between purified TTP and a TNF-alpha ARE sequence [Blackshear et al., 2003; Brewer et al., 2004; Cao, 2004]. Based on a gel shift assay, recombinant TTP purified from *E. coli* and recombinant TTP transfected into mammalian HEK293 cells bound to a TNF-alpha ARE sequence with an affinity of 13–18 and 28–36 nM, respectively [Cao, 2004]. Also, a 73 amino acid synthetic peptide containing a TTP tandem zinc finger domain bound to a TNF-alpha ARE sequence with a K_d of 10 nM by gel shift and 19 nM by fluorescence anisotropy [Blackshear et al., 2003; Brewer et al., 2004]. In contrast to the high affinity binding to the GM-CSF, IL-3, TNF-alpha, IL-2, and c-fos AU-rich sequences that we observed, the TTP-containing complex did not appear to bind to the c-myc, M1, or M8 sequences, demonstrating that the binding interaction was sequence specific.

Gel shift experiments were also performed using S100 extracts from TTP-transfected HeLa cells and the radiolabeled GM-CSF AU-rich probe (Fig. 7). As was seen using total cytoplasmic extracts, the TTP-containing complex was specifically competed with unlabelled GM-CSF, IL-2, or TNF-alpha AU-rich sequences but was not competed with c-myc or M8 sequences. Unlike the competitive binding assay in total cytoplasmic extracts which showed only a single shifted complex (Fig. 5), an additional minor band (labeled with an asterisk in Fig. 7) was seen when S100 extracts were used. This additional band was probably due to the presence of an unknown ARE-binding activity that localized to the S100 fraction. Despite the presence of this minor band, the TTP-containing complex in S100 extracts displayed similar binding characteristics to the complex observed in total cytoplasmic extracts.

TTP Binding Correlates With the Enhanced Decay of ARE-Containing Transcripts in a Cell-Free System

We have shown that TTP binds to AU-rich RNA as a multi-protein complex and recruits components of the mRNA decay machinery to the RNA. We have also characterized the RNA-binding specificity of the TTP-containing complex. We hypothesize that TTP functions by

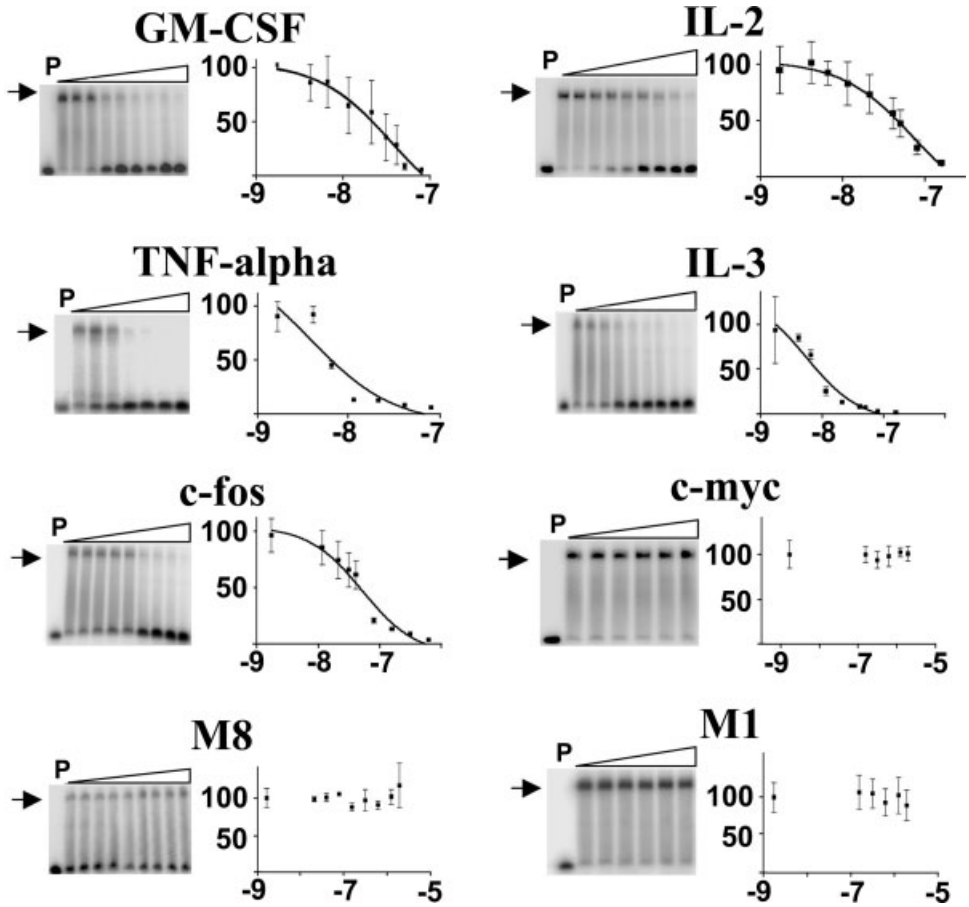

Fig. 5. TTP in cytoplasmic extracts binds with high affinity to a variety of ARE sequences. Cytoplasmic extracts from TTP-transfected HeLa cells (10 μg of protein) were incubated with 35 fmol of a ^{32}P end-labeled GM-CSF RNA probe in the presence of increasing amounts of unlabeled GM-CSF (50 fmol–1.6 pmol), IL-2 (50 fmol–3.2 pmol), TNF-alpha (50 fmol–1.6 pmol), IL-3 (50 fmol–3.2 pmol), c-fos (200 fmol–12.8 pmol), c-myc (3.2–40 pmol), M8 (400 fmol–40 pmol) or M1 (3.2–40 pmol) RNA oligonucleotides. Binding reactions were then separated by electrophoresis on 5% polyacrylamide gels under non-denaturing conditions. The bands representing the free probe and the TTP-containing RNA-protein complex were visualized and quantified using a phosphorimager. The first lane of each gel contains probe alone (P). The position of migration of the TTP-containing complex is indicated with an arrow. The graph to the right of each gel represents binding data from three identical independent experiments. Percent maximal binding (Y-axis) was plotted against the log of the total RNA concentration in molar (X-axis) using Graph Pad Prism version 4.03 Software. Each point represents the mean and standard error of the mean (SEM) from three experiments.

binding to specific ARE sequences and facilitates mRNA decay by physically recruiting mRNA components such as PM-scl75 and Xrn1 to the transcript so that they can carry out their exonucleolytic activities. We therefore predicted that AU-rich sequences that serve as high affinity binding sites for TTP-containing complexes would function to target mRNA for decay. We used a cell free mRNA decay assay based on HeLa S100 extracts that recapitulates known aspects of RNA turnover [Fritz et al., 2000] to test this prediction. We prepared S100 extracts from mock-transfected and TTP-transfected HeLa cells and performed Western blotting to confirm that transfected TTP localizes to the S100 extract (Fig. 8A). Capped, polyadenylated, radiolabeled mRNA transcripts were prepared that contained the identical short AU-rich sequences from GM-CSF, TNF-alpha, IL-2, c-myc, and M8 that were used in our competitive binding experiments. These radiolabeled transcripts were used as substrates in the cell free decay assay, and their decay was analyzed over a 4-h time course (Fig. 8B). A small amount of baseline decay was noted for all of the transcripts tested when S100 extracts from mock-transfected HeLa cells were used (left panels). Compared to the minimal

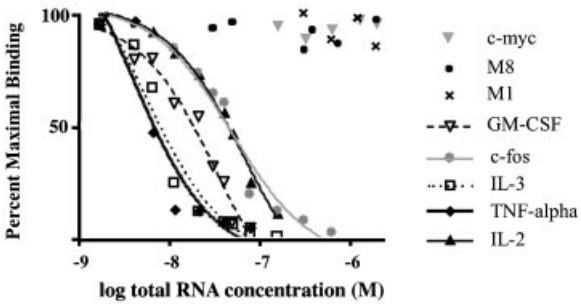

Fig. 6. A variety of ARE sequences compete for binding by TTP. The graphs shown in Figure 5 were combined into one graph. Error bars were omitted to allow better visualization of the data.

deadenylation and decay observed in S100 extracts from mock-transfected cells (lanes 2–4), the GM-CSF, TNF-alpha, and IL-2 ARE-containing transcripts all exhibited markedly accelerated deadenylation in S100 extracts from TTP-transfected cells, as evidenced by the appearance of deadenylated decay intermediates (lanes 6–8). The finding that overexpression of TTP led to the accumulation of deadenylated decay intermediates suggests that TTP had a pronounced effect on the deadenylation of these transcripts but only a modest effect on the decay of the message bodies. Overexpression of TTP had very little effect on the deadenylation or decay of transcripts that did not contain TTP-binding sites such as the M8 transcript or the transcript that contained a U-rich region from the c-myc ARE. These results suggest that presence of high affinity TTP-binding sites correlates with the ability of TTP to enhance transcript deadenylation. This correlation may be due to TTP-dependent recruitment of functional components of the decay machinery including Xrn1 and PM-scl75.

DISCUSSION

TTP is an ARE-binding protein that enhances the deadenylation and decay of ARE-containing transcripts. The role of TTP in mediating mRNA decay in vivo has been established in a TTP knockout mouse model for the ARE-containing transcripts TNF-alpha, GM-CSF, and IL-2 [Carballo et al., 1998, 2000; Ogilvie et al., 2005]. Clues about the molecular mechanisms by which TTP functions to mediate mRNA decay are surfacing. TTP has been shown to interact with components of the cellular mRNA degradation machinery including the exosome, the 5′–3′ exonuclease Xrn1, the deadenylase Ccr4 and the decapping enzyme Dcp1 [Chen et al., 2001; Lykke-Andersen and Wagner, 2005]. These interactions were characterized in an RNA-independent fashion, but led to the development of a model whereby TTP functions by recruiting components of the cellular mRNA decay machinery to ARE-containing transcripts. Our finding that the exonuclease Xrn1 and the exosome component PM-scl75 are part of a TTP-containing complex that binds specifically to ARE sequences supports this model. Based on previous reports that TTP associates with Dcp1 [Lykke-Andersen and Wagner, 2005] and that TTP activates PARN activity [Lai et al., 2003], we may have expected to find these proteins in our TTP-containing complex. In our gel shift experiments, however, we did not find Dcp1 or PARN to be associated with ARE-containing RNA or to be part of the TTP-containing complex based on super-shift experiments with anti-Dcp1 or anti-PARN antibodies (data not shown). Also, antibodies against components of miRNA processing and function, including dicer and argonaute, did not supershift the TTP-containing complex (data not shown). These negative results may be due to

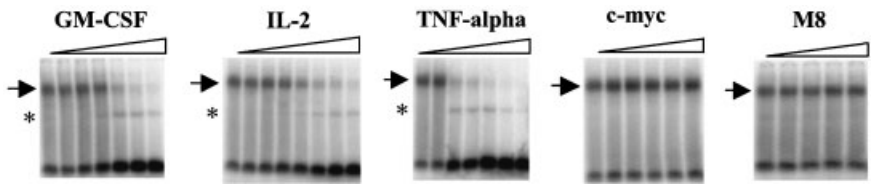

Fig. 7. TTP localized to cytoplasmic S100 extracts binds specifically to ARE sequences. Cytoplasmic S100 extracts from TTP-transfected HeLa cells (10 μg of protein) were incubated with 35 fmol of a ^{32}P end-labeled GM-CSF RNA probe in the presence of increasing amounts of unlabeled GM-CSF (50 fmol–1.6 pmol), IL-2 (50 fmol–3.2 pmol), TNF-alpha (50 fmol–1.6 pmol), c-myc (3.2 pmol–40 pmol) or M8 (400 fmol–40 pmol) RNA oligonucleotides. Reaction mixtures were then separated by electrophoresis on 5% polyacrylamide gels under non-denaturing conditions. The positions of migration of the TTP-containing RNA-protein complex are indicated with arrows. The position of migration of a minor unidentified band is indicated with an asterisk.

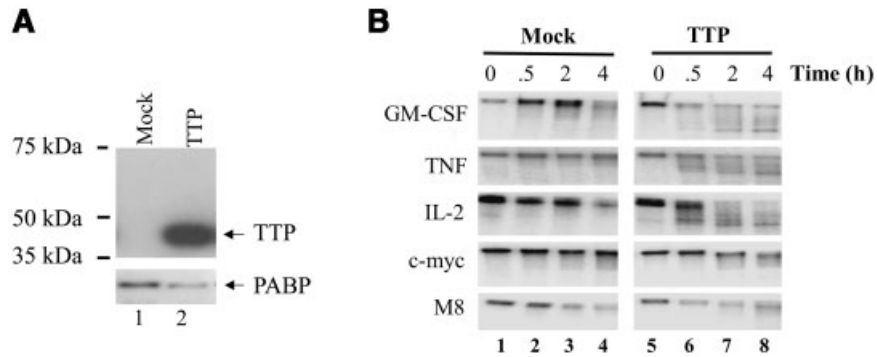

Fig. 8. TTP promotes the specific decay of short ARE-containing transcripts in S100 extracts. HeLa cells were transiently transfected with 10 μg of the pcDNA-3 (mock) or HA-pCMV.TTP.Tag (TTP) plasmids. S100 cytoplasmic extracts were prepared 48 h later. **A**: Western blot analyses were performed on S100 extracts (25 μg of protein) from mock and TTP transfected HeLa cells using an anti-HA antibody to detect HA-tagged TTP and an anti-poly A binding protein antibody to ensure equal loading. The TTP and poly A binding protein (PABP) bands are designated by arrows. **B**: Decay of polyadenylated RNA substrates was assessed using S100 extracts from mock-transfected (Mock) and TTP-transfected (TTP) HeLa cells. S100 extracts (25 μg of protein) were mixed in a cell free decay assay with 25 fmol of ^{32}P-internally labeled, capped, polyadenylated transcripts that contained GM-CSF, TNF, IL-2, c-myc, or M8 sequences. Reaction mixture aliquots were taken at 0, 0.5, 2, and 4 h time points and examined for decay. RNAs were isolated and separated by electrophoresis on 10% polyacrylamide urea gels and radiolabeled bands were visualized using a phosphorimager. The fully adenylated, non-decayed transcripts are sharp bands at time zero for each condition tested (**lanes 1** and **5**).

transient or unstable interactions between TTP and these proteins, dissociation of the TTP-containing complex during the gel shift procedure, or an inability of the antibodies to recognize these proteins in the context of the multiprotein complex. In extracts from mock-transfected HeLa cells, we did not observe an association of Xrn1 or PM-scl75 with ARE sequences, but only observed this association in TTP-transfected cells, suggesting that the recruitment of these proteins to the RNA depended on the presence of TTP. Based on our results, it is not apparent if TTP binds first to the RNA and then recruits PM-Scl75 and Xrn1 or if these proteins pre-assemble with TTP and then bind to the RNA as a pre-formed complex. Given that these proteins can associate with TTP in an RNA-independent manner [Lykke-Andersen and Wagner, 2005], it seems more likely that TTP binds to RNA as a pre-formed complex.

Since TTP binds to RNA as a multiprotein complex, we used a gel shift competitive binding assay using TTP-containing cytoplasmic extracts to assess the binding affinity of the TTP-containing complex for various AU-rich or U-rich sequence. We found that TTP bound with high apparent affinities to the 31 nucleotide AU-rich sequences from the TNF-alpha transcript or the 32 nucleotide AU-rich sequence from the IL-2 transcript. We have shown that these short sequences are sufficient for TTP-mediated mRNA decay when they are inserted into the 3′ UTR of a beta-globin reporter transcript (Fig. 1 and [Ogilvie et al., 2005]). TTP also exhibited high affinity binding to short AU-rich sequences from GM-CSF, IL-3, and c-fos transcripts but poor binding to a U-rich sequence from c-myc, a poly U sequence (M1) or a mutated GM-CSF sequence (M8). Our apparent affinity value of 2 nM for binding by the TTP-containing complex to the TNF-alpha AU-rich sequence is similar to previously reported affinity measurements for recombinant TTP or TTP peptides for TNF-alpha AU-rich sequences which ranged from 10 to 36 nM [Blackshear et al., 2003; Brewer et al., 2004; Cao, 2004], although direct comparisons of these affinity measurements cannot be made due to differences in the systems. For our apparent affinity calculations, we assumed that the TTP-containing complex represented a homogenous complex because it was seen as a single predominant band in our gel shift experiments. We cannot exclude, however, the possibility that this band contains heterogenous TTP-containing complexes that have similar electrophoretic mobility.

Our competitive binding data suggested that TTP binds with high affinities to AU-rich sequences from GM-CSF, IL-3, TNF-alpha, IL-2, and c-fos (apparent K_d ranging from

2–44 nM), although minor differences in the affinity for these sequences could not be evaluated due to the level of variability in our data. These sequences contained a large sampling of potential AU-rich motifs (see Table I), and all of them contained at least one AUUUA pentamer in the context of other AU-rich sequences. The AU-rich sequences from GM-CSF, IL-3, and TNF-alpha contained the UUAUUUAUU nonamer sequence identified by SELEX as a TTP-binding sequence [Worthington et al., 2002], but the sequences derived from IL-2 and c-fos did not. Clearly, TTP has some flexibility in its sequence requirements for binding and future detailed mutagenesis would be necessary to define the precise sequence requirements for TTP binding.

Using a cell-free mRNA decay assay, we demonstrated that the presence of short AU-rich sequences that bound to the TTP-containing complex with high affinity correlated with a TTP-dependent acceleration in deadenylation and mRNA decay. This TTP-dependent acceleration in decay was not seen when sequences that lacked high affinity TTP binding sites were used. These cell-free mRNA decay experiments were performed in HeLa S100 extracts using the same AU-rich sequences and similar conditions as the binding experiments which showed that TTP had recruited Xrn1 and PM-scl75 to AU-rich RNA (Fig. 3, right), and therefore, it is likely that the TTP-containing complex that forms on AU-rich RNA is functional for promoting the selective decay of ARE-containing transcripts. Our finding that short (22–32 nucleotide) AU-rich high affinity TTP-binding sites are sufficient to allow TTP-dependent mRNA decay supports a model whereby TTP binding is all that is necessary in order for an ARE to be functional. This model is consistent with the finding that TTP is functional in the absence of an ARE if it is tethered to the RNA through a heterologous RNA-binding site [Lykke-Andersen and Wagner, 2005]. This model does not exclude the possibility that TTP also cooperates with other regulatory factors that bind to adjacent sequences within an ARE or to other regulatory elements within a transcript. The fate of a transcript probably depends on the integration of signals from multiple regulatory elements and factors. Although the field is moving forward, the biochemical mechanisms that regulate mRNA decay are still largely unknown. It is likely that additional undiscovered proteins are involved in the deadenylation and decay process. Further characterizing the function of TTP and the other factors involved in ARE-mediated mRNA decay using the cell free decay assay will further elucidate the biochemical mechanisms of mRNA decay.

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