**HuA and Tristetraprolin Are Induced following T Cell Activation and Display Distinct but Overlapping RNA Binding Specificities**

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Arvind Raghavan†, Rachel L. Robison§, Jennifer McNabb‡, Cameron R. Miller¶, Darlisha A. Williams§, and Paul R. Bohjanen¶§**

From the †Department of Microbiology, Department of Medicine, and §Microbiology, Immunology and Cancer Biology Graduate Program, University of Minnesota, Minneapolis, Minnesota 55455 and the ¶Division of Infectious Diseases and International Health, Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710

AU-rich elements found in the 3′-untranslated regions of cytokine and proto-oncogene transcripts regulate mRNA degradation and function as binding sites for the mRNA-stabilizing protein HuA and the mRNA-destabilizing protein tristetraprolin. Experiments were performed to evaluate the expression of HuA and tristetraprolin in purified human T lymphocytes and to evaluate the ability of these proteins to recognize specific AU-rich sequences. HuA is a predominantly nuclear protein that can also be found in the cytoplasm of resting T lymphocytes. Within 1 h after stimulation of T lymphocytes with anti-T cell receptor antibodies or a combination of a phorbol myristate acetate and ionomycin, an increase in cytoplasmic HuA RNA-binding activity was observed. Although absent in resting cells, cytoplasmic tristetraprolin protein was detected 3–6 h following activation. HuA recognized specific AU-rich sequences found in c-jun or c-myc mRNA that were poorly recognized by tristetraprolin. In contrast, tristetraprolin recognized an AU-rich sequence in interleukin-2 mRNA that was poorly recognized by HuA. Both HuA and tristetraprolin, however, recognized AU-rich sequences from c-fos, interleukin-3, tumor necrosis factor-α, and granulocyte/macrophage colony-stimulating factor mRNA. HuA may transiently stabilize a subset of AU-rich element-containing transcripts following T lymphocyte activation, and tristetraprolin may subsequently mediate their degradation.

Immune cellular activation, proliferation, and effector function require precise control of growth regulatory genes. For example, T lymphocyte activation induces transient expression of a defined pattern of early response growth regulatory genes, including proto-oncogenes and cytokine genes (1). After normal cellular activation, these growth regulatory genes are expressed for a defined period and then their expression is turned off. Failure to turn off the expression of many of these genes has been associated with malignancy (2, 3). The molecular mechanisms by which expression of early response genes are turned off includes selective degradation of early response gene mRNA. Many of these mRNA transcripts contain conserved AU-rich elements (AREs)1 in their 3′-untranslated region (UTR), which allow these transcripts to be distinguished from other transcripts and thereby be selectively regulated (4, 5). These AREs have been implicated as selective regulators of mRNA localization (6), translation (7, 8), and degradation (5). Thus, a given ARE may serve multiple regulatory functions. The observation that AREs function as instability elements was first demonstrated by Shaw and Kamen in 1986 (5), when they showed that introduction of a 51-nucleotide AU-rich sequence from the granulocyte/macrophage colony-stimulating factor (GM-CSF) 3′-UTR into the 3′-UTR of rabbit β-globin mRNA conferred instability on the otherwise stable β-globin mRNA. Since then, AREs from numerous early response gene transcripts have been shown to function as instability elements (for reviews, see Refs. 9–11) and have been classified according to their sequence features and degradation kinetics (11, 12). Class 1 AREs contain dispersed copies of the sequence motif AUUUA found in the context of other U-rich sequences. This class of AREs is found mostly in transcripts from nuclear transcription factors and proto-oncogenes such as c-fos or c-myc. Class II AREs contain tandemly reiterated copies of the AUUUA sequence motif such as is found in the 3′-UTRs from the cytokine genes tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), interleukin-3 (IL-3), and GM-CSF. In contrast, class III AREs, such as the ARE from c-jun, do not contain the AUUUA motif but rather contain other U-rich sequences. Although AREs appear to target early response gene transcripts for degradation, ARE-containing transcripts may be differentially regulated based on the class of ARE they contain (12).

The mechanism by which AREs regulate mRNA degradation is largely unknown but is thought to involve trans-acting factors that selectively regulate the deadenylation and subsequent degradation of ARE-containing transcripts (10, 11). Numerous trans-acting factors have been identified in crude cellular extracts that bind to AU-rich RNA sequences in vitro (13–18), and several of these correspond to cloned genes (19–25). The function of only a small subset of these proteins has been examined. One of these proteins, AUF-1, binds to the AREs from a variety of genes, including cytokine genes and proto-oncogenes (17), and the binding affinity of recombinant AUF-1 for a panel of AU-rich sequences correlated with the ability of these sequences to function as instability elements (26).

1 The abbreviations used are: ARE, AU-rich element; UTR, untranslated region; TNF-α, tumor necrosis factor-α; IL-2, interleukin-2; IL-3, interleukin-3; GM-CSF, granulocyte-macrophage colony-stimulating factor; TTP, tristetraprolin; P+1, phorbol myristate acetate and ionomycin; α-CD3, anti-α T cell receptor antibody.
Members of the ELAV family of RNA-binding proteins also bind to AREs. This family consists of the tissue-specific RNA-binding proteins HuB, HuC, and HuD, as well as the ubiquitously expressed protein HuA (27, 28). HuA is a predominantly nuclear RNA-binding protein that shuttles between the nucleus and the cytoplasm (29). Recombinant HuA has been shown to stabilize ARE-containing transcripts in vitro (30), and overexpression of HuA (31–33) or HuB (34) results in stabilization of ARE-containing transcripts in vivo. Tristetraprolin (TTP) is another cloned ARE-binding protein that appears to play a role in ARE-mediated mRNA degradation. TNP-α is overproduced in macrophages from TTP knock-out mice through stabilization of TNF-α mRNA, and overexpression of TTP results in decreased accumulation of ARE-containing transcripts (25, 35), suggesting that TTP may mediate degradation of these transcripts. Because HuA and TTP have opposite effects on the stability of ARE-containing transcripts, it is possible that their opposing activities may constitute a homeostatic post-transcriptional mechanism to control gene expression.

Our work has focused on understanding the role of mRNA stability in the regulation of T lymphocyte activation. Experiments were performed to examine expression of HuA and TTP in purified human T lymphocytes and to examine the ability of these proteins to bind to a variety of T lymphocyte-derived AU-rich sequences from early response gene transcripts. We found that, although HuA was present in cytoplasmic extracts from resting T lymphocytes, its RNA-binding activity increased rapidly, within 1 h, following T lymphocyte activation. In contrast to HuA, TTP was not present in cytoplasmic extracts from resting T lymphocytes. TTP was induced following T lymphocyte activation later than HuA, with detectable protein levels occurring 3–6 h after T lymphocyte activation. We also found that HuA and TTP had distinct but overlapping RNA-binding specificities. In particular, HuA and TTP both bound to AU-rich sequences found in the 3’-UTR of the T lymphocyte transcripts c-fos, IL-3, TNF-α, and GM-CSF. Following T lymphocyte activation, HuA may bind to these transcripts early, thereby stabilizing them and allowing them to be expressed, and TTP may subsequently displace HuA and target them for degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Purification and Culture**—Human peripheral blood mononuclear cells were isolated from buffy coat white blood cell packs obtained from the American Red Cross by centrifugation through a Ficoll-Hypaque (Amersham Biosciences, Inc.) cushion. Red blood cells were further removed by hypotonic lysis, and monocytes and B cells were removed based on their binding to anti-immunoglobulin-coated glass beads using T cell enrichment columns (R&D Systems). The resulting cell population usually consisted of 90–95% T lymphocytes based on flow cytometry using anti-T cell receptor (anti-CD3) antibodies. These cells were cultured overnight in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. In some experiments, T lymphocytes were stimulated with 10 ng/ml phorbol myristate acetate and 0.4 μM ionomycin (P+I) or with 1 μg/ml immobilized anti-CD3 antibody (R&D Systems) with or without 1 μg/ml soluble anti-CD28 antibody (R&D Systems) as described previously (13, 15).

HeLa cells were grown in monolayers in Iscove’s modified Dulbecco’s medium supplemented with 10% bovine calf serum, 2 mM l-glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

**Preparation of Cytoplasmic Extracts—**Cytoplasmic extracts were prepared by lysing cells in a buffer containing 0.2% Nonidet P-40, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM MgCl₂, 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, and cytoplasmic extracts were immediately frozen on dry ice and were stored at −80 °C. The protein concentration of the extracts was determined by a colorimetric assay using a commercially available reagent (Bio-Rad) according to manufacturer’s instructions.

**RNA Probes and Competitors**—Unless indicated otherwise, RNA transcripts were synthesized from linearized plasmid templates using T7 RNA polymerase as described previously (15). Radiolabeled transcripts were prepared by inclusion of [α-32P]UTP (3000 Ci/mmol; Amersham Biosciences, Inc.) in the reaction, and the resulting RNA had a specific activity of ~8 × 10⁶ cpm/μg. After cleavage with ribonuclease T1, the GM-CSF ARE probe had the sequence AUUUUAAUUUUAAUUUUAAUCUG, and the M1 probe had the sequence UUUUUUUUUUUUUUUUUUUUUCUG. RNA oligonucleotides were purchased commercially (Dharmacon Research) and were used as probes or competitors in some experiments. Table I shows the sequences of the RNA oligonucleotides used. These RNA oligonucleotides were gel-purified and quantified based on their optical density at 260 nm. In some experiments, radiolabeled RNA oligonucleotides were end-labeled with [γ-32P]ATP (6000 Ci/mmol) using T4 polynucleotide kinase, producing probes with a specific activity of ~4 × 10⁶ cpm/μg.

**RNA-Protein Ultraviolet Cross-linking Assay—**Cytoplasmic extracts were incubated with a radiolabeled RNA probe at room temperature for 30 min in a buffer containing 0.2% Nonidet P-40, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM MgCl₂, 1 mM dithiothreitol, and 5% glycerol in the presence of 1 unit/μl ribonuclease T1 (Calbiochem) and 5 mg/ml heparan sulfate (Sigma). Unless indicated otherwise, each reaction contained 8 μg of cytoplasmic protein and 8 fmol of radiolabeled RNA probe in a total volume of 24 μl. Ribonuclease T1 was omitted from the reaction if end-labeled RNA and cold oligonucleotides were used. The reaction mixtures were irradiated with 254-nm ultraviolet light using a Stratagene ultraviolet (UV) cross-linking apparatus (Stratagene) and were separated on SDS-polyacrylamide gels. The gels were dried and were analyzed on a PhosphorImager (Molecular Dynamics).

**Antibodies and Immunoprecipitation—**The anti-HuA antiserum (36) was obtained from Dr. Jack D. Keene at Duke University Medical Center. This antiserum was generated by immunizing a rabbit with recombinant murine HuA protein. Pre-immune serum from this rabbit was used as a control. The anti-TTP antiserum was obtained from Dr. Perry J. Blackshear (NIEHS, National Institutes of Health). This antisera was derived from a rabbit that was immunized against recombinant human TTP. These antibodies were coated onto protein A-Sepharose beads (Sigma), and the beads were washed. RNA-protein UV cross-linking reactions were performed as described above and were incubated with antibody-coated beads for 2 h on a rotator at 4 °C in a buffer containing 2% Nonidet P-40, 0.1% SDS, 40 mM KCl, 10 mM HEPES (pH 7.9), 3 mM MgCl₂, 1 mM dithiothreitol, and 5% glycerol. The beads were separated from the supernatants by centrifugation, and the beads were washed four times in a buffer containing 150 mM NaCl, 50 mM Tris (pH 7.8), 1% Nonidet P-40, and 0.1% SDS. Material from the supernatants or the beads was separated on SDS-polyacrylamide gels and analyzed by autoradiography on film or a PhosphorImager.

**Northern Hybridization—**Total cellular RNA was prepared from unstimulated or stimulated human T cells using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s directions. The RNA samples were separated by electrophoresis on formaldehyde-agarose gels (20 μg of RNA per lane) and were blotted onto Duralon membranes (Stratagene). The membranes were hybridized sequentially with a 32P-labeled 1.4-kb murine TTP cDNA probe (25) and a glyceraldehyde-3-phosphate dehydrogenase DNA probe (Ambion). After hybridization, blots were washed and analyzed using a PhosphorImager.

**Western Blotting—**Cytoplasmic extracts containing 30 μg of protein were separated by electrophoresis and were electroblotted onto Immobilon P membranes (Millipore). These membranes were probed with an anti-TTP antiserum or with a commercially available anti-actin antibody (Amersham Biosciences). The ECL system (Amersham Biosciences, Inc.) was used to visualize antibody binding to the membrane.

**Transient Transfection—**HeLa cells grown in monolayers in tissue culture plates were transfected for 6 h using the Lipofectin reagent (Life Technologies, Inc.) with the pCMV.TTP.Tag expression plasmid, which encodes the full-length human TTP cDNA linked to a hemagglutinin tag (25). The pCDNA-3 plasmid (Invitrogen) or the pCH110 ribonuclease T1 expression plasmid (Amersham Biosciences, Inc.) were used as mock controls. After an additional 24 h, the cells were harvested, cytoplasmic extracts were prepared, and RNA-protein UV cross-linking assays were performed as described above.

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unstimulated T lymphocytes were incubated with a radiolabeled GM-CSF ARE probe and were then treated with UV radiation. These reactions were performed with extracts from unstimulated T lymphocytes and the cytoplasmic extracts from unstimulated T lymphocytes and the GM-CSF ARE probe. After UV cross-linking, immunoprecipitation experiments were carried out to determine whether the AU-A activity was attributable to the HuA protein. UV cross-linking reactions were performed using cytoplasmic extracts from unstimulated T lymphocytes and the GM-CSF ARE probe. After UV cross-linking, immunoprecipitations were performed with control, anti-HuA-coated, or anti-TTP-coated beads. As seen in Fig. 1B, anti-HuA-coated beads specifically immunoprecipitated AU-A from extracts from unstimulated T lymphocytes (lane 5), whereas beads coated with pre-immune rabbit serum (lane 4) or anti-TTP antiserum (lane 6) did not.

RESULTS

HuA Is Present in T Lymphocyte Cytoplasmic Extracts—A set of ARE-binding activities, termed AU-A, AU-B, and AU-C, were identified previously in cytoplasmic extracts from purified human T lymphocytes using an RNA-protein UV cross-linking assay (13, 15). As seen in Fig. 1A, the AU-A activity present in cytoplasmic extracts from unstimulated T lymphocytes was found to UV cross-link to an AU-rich sequence found in the 3′-UTR of GM-CSF mRNA (lane 1), whereas the AU-B and AU-C activities were found only in extracts from activated T lymphocytes (lane 7). AU-A has striking biochemical similarities to the HuA protein; both are ubiquitously expressed proteins that shuttle between the nucleus and the cytoplasm (13, 29, 37), and they have similar molecular weights and RNA-binding specificities (13, 14, 24). Therefore, immunoprecipitation experiments were carried out to determine whether the AU-A activity was attributable to the HuA protein. UV cross-linking reactions were performed using cytoplasmic extracts from unstimulated T lymphocytes and the GM-CSF ARE probe. After UV cross-linking, immunoprecipitations were performed with control, anti-HuA-coated, or anti-TTP-coated beads. As seen in Fig. 1B, anti-HuA-coated beads specifically immunoprecipitated AU-A from extracts from unstimulated T lymphocytes (lane 5), whereas beads coated with pre-immune rabbit serum (lane 4) or anti-TTP antiserum (lane 6) did not. In addition, anti-HuA-coated beads specifically depleted AU-A from the supernatants (lane 2). Specific and quantitative depletion of AU-A from UV cross-linking reactions performed with extracts from unstimulated T lymphocytes was observed in three independent experiments. The marked similarities between AU-A and HuA (13, 14, 24, 29, 37) together with the immunoprecipitation data presented here strongly suggest that the AU-A RNA-binding activity can be attributed to the HuA protein.

Cytoplasmic HuA RNA-binding Activity Is Induced following T Lymphocyte Activation—Experiments were performed to determine whether cytoplasmic HuA activity was regulated following T lymphocyte activation. The AU-A (HuA) RNA-binding activity could be distinguished from the AU-B and AU-C RNA-binding activities based upon different RNA-binding specificities. As seen in Fig. 1A (lanes 2–6), AU-A (HuA) activity present in extracts from unstimulated cells was competed with a 22-nucleotide poly(U) sequence (M1 RNA). In contrast, concentrations of competitor M1 RNA that completely inhibited AU-A (HuA) binding had little or no effect on binding by AU-B or AU-C (compare lanes 4–6 to lanes 10–12). Thus, the M1 sequence could be used as a radiolabeled probe to assess HuA binding in isolation, without interference from the AU-B or AU-C activities. As seen in Fig. 2A, HuA RNA-binding activity in cytoplasmic extracts was increased 6 h after T lymphocyte stimulation with a combination of phorbol myristate acetate and ionomycin (P+I) or with an anti-T cell receptor antibody (α-CD3). Evaluation of actin expression by Western blot in duplicate samples demonstrated that the increase in HuA activity was not because of an error in sample quantification (lower panel). The increase in HuA activity following T lymphocyte activation has been observed in more than 10 independent experiments. Interestingly, the increase in cytoplasmic HuA activity occurred very rapidly, within 1 h, following T lymphocyte stimulation with P+I (Fig. 2B) or anti-CD3 (data not shown). The induced RNA-binding activity in the cytoplasm seen in Fig. 2A (A and B) is attributable to HuA because all of the induced activity was immunoprecipitated and immunodepleted by anti-HuA serum (Fig. 2C). Because HuA is known to shuttle between the nucleus and the cytoplasm (29), it is possible that the rapid increase in cytoplasmic HuA activity is caused by redistribution of HuA from the nucleus to the cytoplasm.

TTP Is Induced following T Lymphocyte Activation—Given the inducible expression of TTP in macrophages and its role in regulating TNF-α and GM-CSF expression (25, 35, 38), we postulated that TTP could also be a regulator of cytokine expression in T lymphocytes. Therefore, experiments were performed to evaluate TTP mRNA expression in T lymphocytes. Total cellular RNA from T cells that were unstimulated or were...
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Fig. 2. Induction of HuA following T lymphocyte activation. A, cytoplasmic extracts from T lymphocytes that were treated for 6 h with medium alone (MED), P+I, or α-CD3 were incubated with radiolabeled M1 RNA (see Table I). The reaction mixtures were treated with UV radiation and were separated by electrophoresis on 10% SDS-polyacrylamide gels (top panel). The arrow labeled HuA indicates the position of migration of the HuA-RNA complex. Duplicate samples were analyzed by Western blot for expression of actin to ensure equal loading (bottom panel). The migration of protein molecular size markers is shown to the left of each panel in kilodaltons. B, cytoplasmic extracts from T lymphocytes that were treated for 0, 1, 3, or 6 h with P+I were incubated with radiolabeled M1 RNA. The reaction mixtures were treated with UV radiation and were separated by electrophoresis on 10% SDS-polyacrylamide gels (top panel). The arrow labeled HuA indicates the position of migration of the HuA-RNA complex. Duplicate samples were analyzed by Western blot for expression of actin to ensure equal loading (bottom panel). C, cytoplasmic extracts from unstimulated (MED) or P+I-stimulated T lymphocytes were incubated with radiolabeled M1 RNA and then were treated with UV radiation. These reactions were then mixed with protein A-Sepharose beads that had been coated with control rabbit serum (Control), anti-HuA antiserum (αHuA), or anti-TTP antiserum (αTTP). The beads were removed by centrifugation and washed. One-eighth of the material in the supernatants (SUPS) or one half of the material on the beads (BEADS) was separated by electrophoresis on a 10% SDS-polyacrylamide gel.

stimulated for 30, 60, or 90 min with P+I was used to prepare Northern blots that were hybridized with a murine TTP cDNA probe. Although only low levels of TTP mRNA were observed in unstimulated T cells, TTP mRNA was rapidly induced within 30 min of stimulation and was decreasing toward basal levels within 90 min (Fig. 3A). TTP mRNA levels were also induced by anti-CD3 stimulation (data not shown). The time course of induction of HuA in T lymphocytes is similar to the induction of TTP mRNA in macrophages following stimulation with TNF or lipopolysaccharide (25). Western blot experiments were also performed to determine whether the increased TTP mRNA levels correlated with increased TTP protein expression. As seen in Fig. 3B, T cell activation for 6 h with P+I or a combination of anti-CD3 and anti-CD28 antibodies led to increased TTP protein expression. TTP protein expression was transient following anti-CD3 stimulation, appearing within 3 h and decreasing to near base-line levels within 12 h (Fig. 3C). The kinetics of TTP expression parallels the expression of cytokine transcripts following T lymphocyte activation (39), raising the possibility that TTP could be a regulator of T lymphocyte cytokine expression.

TTP in T Lymphocyte Cytoplasmic Extracts Has RNA-binding Activity—Because TTP protein was present in cytoplasmic extracts from activated T lymphocytes, experiments were performed to determine whether this TTP protein possessed RNA-binding activity. As was seen in Fig. 1A, P+I stimulation induced the expression of two ARE-binding activities, referred to as AU-B and AU-C, that were detected using a UV cross-linking assay. Unlike HuA (AU-A) activity, these activities were not competed using M1 competitor RNA. UV cross-linking reactions using extracts from P+I-stimulated T lymphocytes and the GM-CSF ARE probe were immunoprecipitated with control, anti-TTP-coated, or anti-HuA-coated beads (Fig. 4A). Anti-HuA-coated beads immunoprecipitated AU-A (lane 5), as was seen previously (Fig. 2), whereas anti-human TTP-coated beads immunoprecipitated two proteins (lane 6) that corresponded to the AU-B and AU-C ARE binding activities (15). An anti-murine TTP antiserum also specifically immunoprecipitated both AU-B and AU-C (data not shown). The comigration of AU-A and AU-B made the experiments shown in Fig. 4A difficult to interpret, a similar experiment was performed in the presence of cold M1 RNA to eliminate AU-A binding (Fig. 4B). Under these conditions, anti-human TTP-coated beads also immunoprecipitated both AU-B and AU-C (lane 8). These results suggest that components of AU-B and AU-C share common epitopes and are consistent with the previous finding that AU-B and AU-C, cross-linked to radiolabeled RNA, have overlapping protease cleavage patterns (15). Because the ratio of the intensities between AU-B and AU-C differs from experiment to experiment, it is possible that AU-B is an in vitro proteolytic product of AU-C. Other investigators have seen two forms of TTP in extracts from stimulated monocyte or fibroblast cell lines: a predominant 43-kDa form and a minor 30-kDa form, which they speculated could be a cleavage product (40). Not all of the AU-B/AU-C RNA-binding activity observed in Fig. 4 can be attributed to TTP because only a fraction of the AU-C band can be immunodepleted, even with excess TTP antibody (data not shown). Additionally, cytoplasmic extracts from stimulated T lymphocytes from TTP knockout mice retain an RNA-binding activity that resembles AU-C.² We have provisionally designated the anti-TTP component of AU-C as TTP-like factor. Overall, the immunoprecipitation data presented here suggest that at least a component of AU-B/AU-C RNA-binding activity can be attributed to TTP, suggesting that TTP present in cytoplasmic extracts from stimu-

² C. Miller and P. Bohjanen, unpublished result.
lated T lymphocytes is capable of binding to an ARE sequence.

**HuA and TTP Have Distinct but Overlapping RNA-binding Specificities**—A transfection system in HeLa cells was set up to compare the RNA-binding properties of HuA and TTP. HeLa cells were chosen for these experiments because they constitutively express HuA protein (24) but not the TTP protein (Fig. 5B, lane 1). Additionally, HeLa cells express no AU-B or AU-C activity such as is seen in T lymphocytes; therefore, exogenously expressed TTP in HeLa cells can be easily identified. Cytoplasmic extracts were prepared from mock-transfected or TTP-transfected HeLa cells, and UV cross-linking assays were performed using a GM-CSF ARE probe. Mock-transfected HeLa cells expressed HuA as the predominant RNA binding activity (Fig. 5, lane 1). The band labeled HuA in this experiment represents the HuA protein based on specific immunoprecipitation and immunodepletion of this band by anti-HuA antiserum (data not shown). In cytoplasmic extracts from TTP-transfected cells, a new band appeared (lane 5) that represented TTP based on immunoprecipitation with anti-TTP antiserum (TTP) or an anti-actin antibody (Actin). The migration of protein molecular size markers is shown to the left of each panel in kilodaltons. C, Western blots were performed from TTP-transfected HeLa cells (lane 1) or from T lymphocytes that were stimulated for 0, 1, 3, 6, 12, or 24 h with α-CD3 (lanes 2–7). The blots were probed sequentially with anti-TTP antiserum (TTP) or an anti-actin antibody (Actin).

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**Fig. 3. Induction of TTP.** A, Northern blots were performed on total cellular RNA isolated from purified human T lymphocytes that were stimulated with P+I for 0, 30, 60, or 90 min. The same blot was hybridized sequentially with TTP and glyceraldehyde-3-phosphate dehydrogenase probes. Aliquots of each RNA sample were separated on a 1% agarose gel, which was stained with ethidium bromide (EtBr) to ensure equal loading of RNA (top panel). The positions of migration of 28 and 18 S ribosomal RNA are indicated to the right of each panel. B, Western blots were performed from mock-transfected HeLa cells (lane 1), TTP-transfected HeLa cells (lane 2), or T lymphocytes that were stimulated for 6 h with medium alone (lane 3), P+I (lane 4), or anti-CD3 and anti-CD28 antibodies (α-CD3 + α-CD28, lane 5). The blots were probed sequentially with anti-TTP antiserum (TTP) or an anti-actin antibody (Actin). The migration of protein molecular size markers is shown to the left of each panel in kilodaltons. C, Western blots were performed from TTP-transfected HeLa cells (lane 1) or from T lymphocytes that were stimulated for 0, 1, 3, 6, 12, or 24 h with α-CD3 (lanes 2–7). The blots were probed sequentially with anti-TTP antiserum (TTP) or an anti-actin antibody (Actin).
with cold GM-CSF ARE RNA but not cold M1 or M8 RNA. These results suggest that HuA and TTP have different binding specificities, but both recognize the GM-CSF ARE sequence. A panel of competitor RNA oligonucleotides was generated using AU-rich sequences found in the 3′-UTRs of cytokine and proto-oncogene mRNAs (Table I), and these sequences were added to UV cross-linking reactions containing HuA, TTP, and the GM-CSF ARE probe. The HuA and TTP RNA-protein cross-links were both competed efficiently by the c-fos, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities. The HuA RNA-protein cross-link, however, was competed efficiently by the c-jun, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities. The HuA RNA-protein cross-link, however, was competed efficiently by the c-fos, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities. The HuA RNA-protein cross-link, however, was competed efficiently by the c-fos, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities. The HuA RNA-protein cross-link, however, was competed efficiently by the c-fos, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities. The HuA RNA-protein cross-link, however, was competed efficiently by the c-fos, IL-3, and TNF-α sequences, suggesting that HuA and TTP have overlapping RNA binding specificities.

**HuA and TTP Compete for Binding to the GM-CSF ARE Probe**—Because HuA and TTP appeared to have overlapping binding specificities, mixing experiments were performed to determine whether these proteins could compete with each other for binding to a specific RNA sequence. Cytoplasmic extracts from mock-transfected HeLa cells (which contain HuA but not TTP) were mixed with increasing amounts of cytoplasmic extract from TTP-transfected cells (which contain HuA and TTP), and these mixtures were used for UV cross-linking assays with the GM-CSF ARE probe. As seen in Fig. 6, increasing the amount of TTP-containing extract resulted in a progressive decrease in the intensity of the HuA band and a corresponding increase in the TTP band. This result suggests that HuA and TTP competed for the probe. The finding that the TTP band dominated and the HuA band disappeared under conditions where the probe was limiting (lane 5) suggests that TTP has higher affinity for the GM-CSF ARE probe. This finding raises the possibility that in-
duction of TTP following T lymphocyte activation could lead to displacement of HuA bound to certain ARE sequences, thus leading to specific mRNA degradation.

DISCUSSION

Previous work from others suggests that HuA and TTP have opposite effects on mRNA degradation (25, 31–33, 35, 38). Overexpression of HuA has led to stabilization of ARE-containing reporter transcripts (31–33), whereas overexpression of TTP has led to destabilization of reporter constructs (25, 35, 38). Our results suggest that HuA and TTP are both expressed in primary human T lymphocytes and that their expression is regulated by cellular activation. HuA appears to be identical to the AU-A RNA binding activity identified previously in T lymphocytes using an RNA-protein UV cross-linking assay (13). Like all ELAV proteins, HuA contains three RNA recognition motifs and binds to specific AU-rich RNA sequences (23). In resting T lymphocytes, HuA is predominantly nuclear, but is detectable at relatively low levels in the cytoplasm (13). In other cell types, HuA has been shown to shuttle between the nucleus and the cytoplasm and the sequence motifs responsible for shuttling have been characterized (29, 31). It appears that HuA may bind to ARE-containing transcripts in the nucleus and play a role in their transport to the cytoplasm (41). Our finding that levels of cytoplasmic HuA activity rapidly increase following T lymphocyte activation raises intriguing possibilities. The increase in cytoplasmic HuA levels could represent redistribution of HuA from the nucleus to the cytoplasm, new synthesis of HuA, or both. Treatment of murine splenocytes with anti-CD3 antibody or a combination of anti-CD3 plus anti-CD28 antibodies has been shown to lead to an increase in the total amount of cellular HuA 48 h after stimulation, suggesting that new HuA synthesis may occur (36). The increase in cytoplasmic HuA activity observed here, however, occurred very rapidly, within 1 h, after T lymphocyte activation. The rapidity of the increase in cytoplasmic HuA levels seen in our experiments favors redistribution from the nucleus. Perhaps T lymphocyte activation leads to rapid transcription of a bolus of ARE-containing transcripts that subsequently move, along with HuA, from the nucleus to the cytoplasm.

In contrast to HuA, TTP is not present in resting T lymphocytes. Cytoplasmic expression of TTP protein and RNA-binding activity is induced following T lymphocyte activation with highest protein levels occurring ~3–6 h after T lymphocyte activation. By 12 h after activation, TTP levels have returned to near base line. This transient expression of TTP is likely to have regulatory effects on T lymphocyte gene expression. TNF-α is overproduced in lipopolysaccharide-stimulated macrophages derived from TTP knock-out mice through specific stabilization of TNF-α mRNA (25). Additionally, overexpression of TTP leads to decreased accumulation of ARE-containing transcripts, including TNF-α transcripts, suggesting that TTP may bind to and destabilize these transcripts (25, 35). It is possible that TTP serves the same function in T lymphocytes following activation. Perhaps TTP is part of a down-regulatory program whose purpose is to turn off expression of cytokine genes and other early response genes by targeting their mRNA transcripts for degradation.

Although both HuA (15, 23, 42) and TTP (35, 38, 43) have been shown to bind to ARE sequences, our results indicate that these proteins have different RNA binding specificities. For example, HuA binds efficiently to the M1 sequence, but TTP does not. In general, it appears that HuA has a broader RNA binding specificity than TTP because HuA binds to AU-rich sequences (c-fos, IL-2, TNF-α, and GM-CSF), GU-rich sequences (c-jun), or U-rich sequences (M1 and c-myc-1). Interestingly, HuA poorly recognized the AU-rich sequence from IL-2. These results are consistent with the previous finding that overexpression of HuA led to stabilization of reporter RNA constructs containing ARE sequences from c-fos, GM-CSF, or TNF-α (31, 33, 42). In contrast to HuA, TTP binds efficiently only to sequences that contain tandem multimers of the sequence AUUUA, such as c-fos, IL-2, IL-3, TNF-α, and GM-CSF, and TTP binds only poorly to c-jun or c-myc-1 sequences. For some sequences, such as the GM-CSF ARE, HuA and TTP compete for binding. The competition for binding by HuA and TTP to ARE sequences in vivo could lead to specific patterns of gene expression. Because HuA and TTP have different RNA-binding specificities, they may regulate different genes. It is possible that HuA regulates a broad spectrum of ARE-containing transcripts whereas TTP regulates a smaller subset. It is also possible that the different binding specificities of HuA and TTP could explain the differential degradation of ARE-containing transcripts that has been observed (13, 44, 45). Additional ARE-binding factors that have not yet been characterized may also regulate ARE-mediated mRNA decay. For example, a component of the T lymphocyte ARE binding activity AU-C appears to be distinct from TTP. This TTP-like factor is a specific ARE-binding activity that is also induced following T lymphocyte activation. Future work will aim at purifying, identifying, and characterizing this activity.

HuA and TTP may be part of a homeostatic pathway that allows precise regulation of gene expression following T lymphocyte activation. T cell activation induces the transcription of a variety of ARE-containing transcripts that regulate cell growth and function. Following T lymphocyte activation, HuA, which predominates in the nucleus, may bind to a subset of ARE-containing transcripts in the nucleus and mediate their rapid transport to the cytoplasm. Within the cytoplasm, HuA may stabilize these ARE-containing transcripts allowing them to be translated and expressed. Subsequently, TTP expression is induced and the cytoplasmic level of TTP increases. TTP then competes with HuA for the ARE sites on a subset of ARE-containing transcripts. The relative levels and binding affinities of HuA and TTP for specific ARE sequences may determine the fate of these transcripts, with HuA predominance promoting mRNA stabilization and TTP predominance promoting mRNA degradation. At some point, TTP may predominate and facilitate specific mRNA degradation. This model provides a mechanism by which genes that are important in regulating cell growth could be transiently expressed and then precisely turned off at the appropriate time.

The biochemical mechanism by which HuA and TTP regulate mRNA stability is still largely unknown. The process is complex, and it is likely that many of the components of the ARE-mediated mRNA decay pathway have not been identified. An in vitro RNA stability assay was recently described in which ARE-regulated mRNA degradation occurs (30). In this system, addition of recombinant HuA resulted in stabilization of ARE-containing transcripts (30). Perhaps this type of biochemical system can be used to identify and biochemically characterize the components of the ARE-regulated mRNA degradation pathway and to provide a better understanding of the roles of HuA and TTP in regulating mammalian mRNA degradation.

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