Regulation of A + U-rich Element-directed mRNA Turnover Involving Reversible Phosphorylation of AUF1*

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Proteins binding A + U-rich elements (AREs) contribute to the rapid cytoplasmic turnover of mRNAs containing these sequences. However, this process is a regulated event and may be accelerated or inhibited by myriad signal transduction systems. For example, monocyte adherence at sites of inflammation or tissue injury is associated with inhibition of ARE-directed mRNA decay, which contributes to rapid increases in cytokine and inflammatory mediator production. Here, we show that acute exposure of THP-1 monocytic leukemia cells to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate mimics several features of monocyte adherence, including rapid induction and stabilization of ARE-containing mRNAs encoding interleukin-1β and tumor necrosis factor α. Additionally, TPA treatment alters the activity of cytoplasmic complexes that bind AREs, including complexes containing the ARE-specific, mRNA-decay factor, AUF1. Analyses of AUF1 from control and TPA-treated cells indicated that post-translational modifications of the major cytoplasmic isoform, p40AUF1, are altered concomitantly with changes in RNA binding activity and stabilization of ARE-containing mRNAs. In particular, p40AUF1 recovered from polysomes was phosphorylated on Ser\(^{58}\) and Ser\(^{72}\) in untreated cells but lost these modifications following TPA treatment. We propose that selected signal transduction pathways may regulate ARE-directed mRNA turnover by reversible phosphorylation of polysome-associated p40AUF1.

In eukaryotes, cytoplasmic mRNA stability is an important checkpoint in the control of gene expression. Many mRNAs encoding regulatory proteins like cytokines, inflammatory mediators, and oncproteins are constitutively unstable. This ensures that the steady-state levels of these mRNAs, and hence their potential for translation, remain low but also that new steady-state levels are approached quickly following changes in the rate of mRNA synthesis (reviewed in Ref. 1). In mammals, a common feature of many unstable mRNAs is the presence of an A + U-rich element (ARE)\(^3\) within the 3′-untranslated region (3′-UTR). These elements range from 40 to 150 nucleotides in length and exhibit significant variability in sequence composition, but they usually include one or more AUUUA motifs within a U-rich context (2). In general, mRNA turnover mediated by AREs consists of rapid 3′ → 5′ shortening of the poly(A) tail, followed by decay of the mRNA body (3, 4).

The regulation of mRNA decay kinetics by AREs involves their association with any of a number of cellular ARE-binding factors (reviewed in Ref. 5). One such factor, AUF1 (also referred to as heterogeneous nuclear ribonucleoprotein D), is expressed as a family of four protein isoforms resulting from alternative splicing of a common pre-mRNA (6). The larger isoforms, designated by their apparent molecular weights as p42AUF1 and p45AUF1, are largely nuclear (7), probably due to the presence of a binding determinant for components of the nuclear scaffold (8). By contrast, p37AUF1 and p40AUF1 lack this sequence determinant and, as such, may be found in both nuclear and cytoplasmic compartments. AUF1 binding to an ARE is linked to acceleration of mRNA decay, based on extensive studies correlating mRNA turnover rates with AUF1 abundance (9–13) or ARE-binding activity (14, 15). Association of p37AUF1 with an ARE induces the formation of protein oligomers (16) and local conformational changes in the RNA substrate (17), which in turn may recruit additional factors to generate a multisubunit, trans-acting complex on the mRNA (7, 18). Ultimately, the mRNA is degraded by catalytic activities, which may include specific nucleases (19–21) or the proteasome (18). Besides AUF1, other RNA-binding factors have also been implicated in the regulation of mRNA decay rates through AREs. For example, association of tristetraprolin (TTP) with some ARE-containing transcripts enhances their decay (22, 23). By contrast, mammalian factors related to the Drosophila Elav (embryonic lethal abnormal vision) protein, including the ubiquitously expressed HuR and the neuron-specific Hel-N1, are thought to inhibit ARE-directed mRNA

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1 The abbreviations used are: ARE, A + U-rich element; ACN, acetonitrile; actD, actinomycin D; IL-1β, interleukin-1β; IMAC, immobilized metal ion affinity chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; p38MAPK, p38 mitogen-activated protein kinase; RPA, ribonuclease protection assay; TNF-α, tumor necrosis factor α; TPA, 12-O-tetradecanoylphorbol-13-acetate; TTP, tristetraprolin; UTR, untranslated region.
turnover (24–26). Thus, associated trans-acting proteins may modulate the decay kinetics of ARE-containing mRNAs positively or negatively. Additionally, a growing number of ARE-binding proteins have been described to which no specific functions have been ascribed (27–31).

Modulation of ARE-directed mRNA turnover has been observed in response to a plethora of stimuli. For example, the rapid decay of interleukin-3 mRNA in mast cells is inhibited by Ca²⁺ influx (32). Similarly, mRNAs containing AREs are stabilized during heat shock (18). Specific intracellular signaling pathways have also been identified, which contribute to the regulation of ARE-directed mRNA turnover. In particular, components of the p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase pathways are required for stabilization of ARE-containing mRNAs associated with the inflammatory response (33–37) and tumor cell metastasis (38). Both tyrosine kinase and p38MAPK activities are required for the stabilization of interleukin-1β (IL-1β) and GRO mRNAs induced by monocyte adherence (15). By contrast, ARE-dependent stabilization of cyclooxygenase-2 mRNA by Goα coupled receptor signaling in smooth muscle is mediated by the p42/p44 MAP kinases and is independent of p38MAPK activity (39).

Currently, the molecular mechanisms linking activation or inhibition of these signaling pathways to modulation of mRNA decay rates are largely unknown. One possibility is that these systems function by altering the abundance or RNA-binding properties of ARE-binding factors responsible for initiating the decay process. To test this hypothesis, we have examined the stabilization of ARE-containing mRNAs encoding the cytokine IL-1β and the inflammatory mediator tumor necrosis factor α (TNFα), using a cultured cell model that mimics key features of monocyte adherence. In particular, we show that acute exposure of THP-1 monocyte leukemia cells to the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) rapidly induced cell adhesion, accompanied by dramatic increases in levels of IL-1β and TNFα mRNAs. Accumulation of these transcripts included significant mRNA stabilization, similar to that observed following adhesion of primary monocytes (15). Using this model system, we show that the distribution of cytoplasmic ARE binding activities containing AUF1 is altered following TPA treatment, coincident with changes in post-translational modifications of cytoplasmic p40AUF1. Finally, we have identified two sites of phosphorylation on p40AUF1 purified from THP-1 polysomes, which are dephosphorylated following TPA treatment, concomitant with inhibition of ARE-directed mRNA turnover. From these data, we propose that reversible phosphorylation of AUF1 may constitute a critical mechanism for regulating mRNA turnover rates through AREs.

**EXPERIMENTAL PROCEDURES**

**Materials—**THP-1 monocyte leukemia cells were generously provided by Dr. Charles McCull. Oligoribonucleotide substrates encoding the TNFs ARE and a fragment of the rabbit β-globin coding region (Fig. 3A) were synthesized by Dharmacon (Lafayette, CO). Anti-HuR and anti-TPP antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-Hsp70 antibodies were from Stressgen Biotechnology (San Diego, CA). Radiolabeled nucleotides were from ICN (Costa Mesa, CA), and TRIZol reagent was from Invitrogen. Plasmid pGEM12Zf(+) and PolyATtract mRNA Purification System were purchased from Promega (Madison, WI). Actinomycin D (actD) was obtained from Millipore Corp. (Bedford, MA). Acetonitrile (ACN) and GaCl₃ were purchased from Aldrich. Calf intestinal alkaline phosphatase was obtained from Roche Applied Science. Cell Culture—THP-1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and 1 mM L-glutamine in the absence of antibiotics. Defined fetal bovine serum was lot-selected for minimal endotoxin content by HyClone (Logan, UT) and then incubated at 56 °C for 30 min to inactivate residual endotoxins prior to use. mRNA Quantitation by ribonuclease protection assay (RPA) — A plasmid template for synthesis of a riboprobe complimentary to a portion of the IL-1β 3'-UTR was generously provided by Dr. Mary Vermeulen. A cDNA fragment encoding a portion of the TNFα 3'-UTR was amplified by PCR from plasmid pE4 (ATCC, Manassas, VA), and a human β-actin 3'-UTR fragment was amplified by reverse transcription-PCR from THP-1 cell total RNA. The resulting cDNA fragments were then cloned into pGEMTZf (+) to generate plasmid templates for preparation of antisense riboprobes.

5′-End labeled riboprobes used for RPAs were generated in vitro run-off transcription as described previously (40). Riboprobes complementary to IL-1β and TNFα mRNAs were synthesized to specific activities of 1–2 × 10⁶ cpm/µg, whereas β-actin antisense riboprobes were synthesized to 200 cpm/µg. Cellular RNA samples were purified first as the total RNA fraction, extracted using TRIZol reagent according to the manufacturer’s instructions, and then enriched for mRNA using the PolyATtract mRNA purification system. Specific cellular mRNAs were quantified using RPAs (8 µg of poly(A)+ RNA/lane) with RNAses P₁ and T₁, as described previously (41). Protected RNA fragments were fractionated by denaturing gel electrophoresis and were visualized by autoradiography (Amersham Biosciences).

mRNA Decay Assays—Decay rates of IL-1β and TNFα mRNAs were measured by an actD time course assay. Briefly, transcription was inhibited in control or TPA-treated THP-1 cells by the addition of actD (5 µg/ml) to the culture medium, and poly(A)+ RNA samples were harvested at selected time points thereafter. The relative abundances of IL-1β and TNFα mRNAs were determined at each point by RPA and were normalized using β-actin mRNA levels. First- and second-order decay constants (k) were solved by nonlinear regression of the percentage of IL-1β or TNFα mRNA remaining versus time of actD treatment using PRISM version 2.0 (GraphPad, San Diego, CA). Errors about regression solutions (S.E.) were calculated by the software using n = 2–3 degrees of freedom, with replicate experiments yielding similar results. Comparisons of mRNA decay constants for control versus TPA-treated cells were performed using the unpaired t test, with differences exhibiting p < 0.05 considered significant.

Fractionation of THP-1 Cells and Gel Mobility Shift Assays—Nuclear and cytoplasmic fractions of control or TPA-treated (10 nM, 1 h) THP-1 cells were prepared by resuspension (control samples) or scraping (TPA-treated samples) of PBS-washed cells in lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM KCl, 2.5 mM EDTA, and 1% IGEPAL-CA630) containing mixtures of protease inhibitors (1 µg/ml each of leupeptin and pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride) and kinase/phosphatase inhibitors (50 mM sodium fluoride, 20 µM sodium vanadate, 1 µM sodium orthovanadate). Lysis was performed with 7–10 strokes of a loosely fitting Dounce homogenizer and analyzed by phase-contrast microscopy. Nuclei were pelleted by centrifugation at 1000 × g for 10 min and resuspended directly in SDS-PAGE loading buffer for Western analyses. Protein concentrations were determined for cytoplasmic extracts (42).

Cytoplasmic ARE-binding activities were identified in THP-1 cytoplasmic extracts using gel mobility shift assays as described previously (43). Oligoribonucleotide RNA substrates were 5′-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase to specific activities of 3–5 × 10⁶ cpm/µg/ml as described (16). Identical reactions were assembled for antibody supershift assays except that preimmune serum or antiserum to specific monoclonal antibodies was added to a maximum of 10% total reaction volume prior to incubation (16).

**Two-dimensional Western Analyses—**Cytoplasmic extracts (50 µg of protein) from control or TPA-treated THP-1 cells (described above) were loaded onto 1.5 mm × 12 cm isoelectric focusing tube gels in 1 × SDS sample buffer (125 mM Tris-HCl (pH 6.8), 1% SDS, 5% glycerol, 10 mM diithiothreitol, 0.005% bromphenol blue) without heating. Gels containing 30% acrylamide were run in the presence of 1% IGEPAL-CA630 and 0.5% Triton X-100, 0.5%–3% amylolides, 2.3%–7% amylolides, and 2.3%–3% crofides, and were run between 0.1 N NaOH (cathode buffer) and 6 mM H₃PO₄ (anode buffer) for 400 V in 16 h and then 800 V for 2 h. Tube gels were extruded and equilibrated with 1 × SDS sample buffer prior to second dimension fractionation by SDS-PAGE, followed by electroblotting of the Polyvinylidene difluoride membrane and immunodetection of AUF1.

**Analyses of Polyasome-associated p40AUF and Peptide Fragments by Mass Spectrometry—**A ribosomal salt wash was performed by first isolating polyasomes from control or TPA-treated THP-1 cells by hypotonic
mRNA Turnover Regulated by AUF1 Phosphorylation

RESULTS

IL-1β and TNFα mRNAs Are Rapidly Induced and Stabilized in THP-1 Cells following Acute TPA Treatment—Circulating monocytes adhere at sites of infection or tissue injury. This event rapidly triggers the expression of a battery of cytokines and inflammatory mediators, involving both transcriptional and post-transcriptional mechanisms (15, 48). By contrast, THP-1 monocyte leukemia cells grow constitutively in suspension but may become adherent following treatment with phorbol esters. Stimulation of THP-1 cells with phorbol esters like TPA is a popular model of monocytic differentiation to macrophage-like cells, based on the manifestation of adherence, loss of proliferation, phagocytosis, and enhanced production of proinflammatory cytokines in response to lipopolysaccharide (49–52).

Whereas phorbol ester treatment induces THP-1 cells to adopt many macrophage-like characteristics over a period of days, adherence per se may be observed within a mere 15-min exposure to TPA (10 min, data not shown). To determine whether this acute TPA-induced adherence of THP-1 cells was accompanied by increased expression of cytokine and inflammatory mediator mRNAs, similar to that observed in adherent monocytes, the levels of IL-1β and TNFα mRNAs were measured across a time course of TPA treatment (Fig. 1). Increases in IL-1β mRNA levels were observed within 30–60 min and reached a maximum induction of nearly 50-fold within 8 h (Fig. 1, A and C). TNFα mRNA levels were also rapidly increased, attaining 30-fold induction within 4 h (Fig. 1, B and C). In both cases, these increases were transient, with preinduction mRNA levels reached (TNFα) or approached (IL-1β) after 24 h of TPA treatment.

During monocyte adherence, mRNA stabilization contributes to the induction of mRNAs encoding cytokines and other inflammatory mediators (15). To establish whether similar post-transcriptional mechanisms contributed to induction of these mRNAs in the acute TPA-treated THP-1 model, the decay kinetics of IL-1β and TNFα mRNAs were monitored prior to and following TPA-induced cell adherence by actD time course assay (Fig. 2). In all cases, mRNA decay kinetics were well approximated by first-order decay functions (Fig. 2C). Both IL-1β and TNFα mRNAs are relatively unstable in unstimulated THP-1 cells, decaying with half-lives of 29 and 8 min, respectively. However, after 1 h of TPA treatment, both mRNAs were stabilized 6–7-fold (Fig. 2C). In addition, stabilization of each mRNA was sustained as mRNA levels accumulated (Table 1). Taken together, the similarities in mRNA accumulation kinetics, the transient nature of mRNA induction, and the rapid and prolonged stabilization of each mRNA following TPA treatment of THP-1 cells indicate that expression of IL-1β and TNFα mRNAs are coordinately regulated in this system, involving both transcriptional and post-transcriptional mechanisms. As such, these data suggested that acute TPA stimulation of THP-1 cells mimics some features of the adhesion-dependent induction of cytokine and inflammatory mediator mRNA levels in primary monocytes.

The Activity of Cytoplasmic ARE-binding Complexes Including AUF1 Is Modified Concomitant with Inhibition of ARE-directed mRNA Turnover—Several observations indicated the stabilization of IL-1β and TNFα mRNAs in TPA-treated THP-1 cells is probably due to regulation of ARE-directed mRNA turnover. First, these mRNAs are relatively unstable in unstimulated THP-1 cells, decaying with half-lives of 29 and 8 min, respectively. However, after 1 h of TPA treatment, both mRNAs were stabilized 6–7-fold (Fig. 2C). In addition, stabilization of each mRNA was sustained as mRNA levels accumulated (Table 1). Taken together, the similarities in mRNA accumulation kinetics, the transient nature of mRNA induction, and the rapid and prolonged stabilization of each mRNA following TPA treatment of THP-1 cells indicate that expression of IL-1β and TNFα mRNAs are coordinately regulated in this system, involving both transcriptional and post-transcriptional mechanisms. As such, these data suggested that acute TPA stimulation of THP-1 cells mimics some features of the adhesion-dependent induction of cytokine and inflammatory mediator mRNA levels in primary monocytes.

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from control or TPA-treated cells displayed similar migration by gel mobility shift assay, their distribution was significantly different (Fig. 3, B (cf. lane 6 versus lane 2) and C). In particular, the faster mobility complex (complex I) accounts for only 20% of the total ARE substrate bound using extracts from TPA-treated cells, with the remaining 80% contained within the slower mobility complex (complex II). By contrast, the ARE substrate is roughly evenly distributed between these complexes using extracts from control cells.

In order to identify specific ARE-binding proteins within these complexes, antibody supershift assays were performed. Using antiserum specific for AUF1, supershifted complexes were detected in ARE-binding reactions containing cytoplasmic extracts from both control and TPA-treated cells. The addition of anti-AUF1 antiserum to reactions containing control extracts effectively shifted 90% of complex I and 70% of complex II, indicating the presence of AUF1 in both ARE-binding activities (Fig. 3, B (lane 4) and C). In comparable experiments with extracts from TPA-treated cells, complex I was also effectively shifted by anti-AUF1 antiserum (>90%); however, the abundance of complex II was only diminished by ~25% (Fig. 3B, lane 8). Furthermore, increasing the concentration of anti-AUF1 antiserum in the reaction did not significantly increase the fraction of complex II shifted (data not shown). These data indicate that a portion of complex II derived from the cytoplasm of TPA-treated THP-1 cells is not immunoreactive with anti-AUF1 antibodies. This could be the result of conformational differences in complex II between extracts from control or TPA-treated cells, such that AUF1 is largely inaccessible to antibodies in the ribonucleoprotein complex formed using cytoplasmic extracts from TPA-treated cells. Alternatively, complex II may represent a heterogeneous population of ARE binding activities, of which only a small fraction contain AUF1. To evaluate this possibility, additional supershift assays were performed using antibodies specific for the ARE-binding proteins HuR, TTP, and Hsp70; however, no supershifted or antibody-neutralized complexes were detected (data not shown). Whereas this may indicate that these proteins do not associate with the TNF ARE in this system, it is also possible that the epitopes targeted by these antibodies are occluded as a result of interactions between target proteins and RNA or other cytoplasmic components. In any case, differences in the distribution and anti-AUF1 reactivity of cytoplasmic ARE-binding complexes from control versus TPA-treated cells suggest that the abundance and/or RNA-binding activity of cytoplasmic AUF1 is altered in THP-1 cells following TPA treatment.

Post-translational Modifications of p40 AUF1 Are Altered by TPA Treatment—Conceivably, the abundance of cytoplasmic AUF1 could be modified by regulation of either its expression or subcellular distribution. To test these possibilities, Western blots were used to determine whether the level or nucleocytoplasmic distribution of any AUF1 isoform was significantly affected by TPA treatment (Fig. 4A). From THP-1 cells, p37AUF1 and p40AUF1 were recovered almost exclusively in cytoplasmic extracts, whereas p42AUF1 and p45AUF1 were nuclear. Acute exposure to TPA had no detectable effect on either the abundance or subcellular distribution of any isoform of AUF1. Since the overall amount of cytoplasmic AUF1 available

Fig. 1. Transient induction of IL-1β and TNFα mRNAs in THP-1 cells following TPA treatment. THP-1 cells were incubated in medium lacking (0 h) or containing TPA (10 nM) for the times indicated prior to purification of poly(A)+ RNA from suspended (0 h) or TPA-adhered cells (0.5–24 h). Cells lacking TPA were pretreated for 1 h with vehicle (0.1% Me2SO). Relative levels of IL-1β (A) and TNFα (B) mRNAs were evaluated by RPA. β-Actin mRNA was measured to control for sample loading. The marker lanes (probes) contain 5% of the riboprobe added to each sample lane to verify probe excess. The labeled RNA fragment detected in the rRNA lane in A (below the β-actin mRNA protected fragment in the sample lanes) is a relatively stable RNase T1, P1 cleavage product of the IL-1β antisense riboprobe. C, relative levels of IL-1β (circles, solid line) and TNFα (triangles, dashed line) mRNAs normalized to β-actin mRNA levels were plotted as a function of time following TPA treatment.

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for RNA binding did not change following TPA treatment, it follows that some feature(s) of the ARE-binding activity of AUF1 was probably affected, possibly involving post-translational modifications of cytoplasmic AUF1 or modulation of some essential co-factor. The latter hypothesis is less likely, since recombinant AUF1 proteins are capable of binding U-rich sequences in vitro. However, since TPA is a potent activator of signaling pathways involving protein kinase C (55), and AUF1 contains multiple phosphorylation sites (17, 54), it is plausible that TPA might influence AUF1 post-translationally. To test this possibility, two-dimensional Western blots were used to determine (i) whether cytoplasmic AUF1 was post-translationally modified in THP-1 cells and (ii) whether such modifications of AUF1 were changed following TPA treatment. In cytoplasmic extracts from untreated THP-1 cells, several forms of p40AUF1 were detected, which differed in their isoelectric points (Fig. 4B, left), demonstrating that post-translationally modified variants of p40AUF1 were present. By contrast, fewer variants of p40AUF1 were detected in cytoplasmic extracts from TPA-treated cells. In particular, the most acidic form of cytoplasmic p40AUF1 in control cells was undetectable in TPA-treated cells (Fig. 4B, right), indicating that post-translationally modifications of cytoplasmic p40AUF1 are altered by TPA treatment of THP-1 cells. In both control and TPA-treated cells, the concentration of cytoplasmic p37AUF1 was too low to permit accurate assessment of post-translational modifications by this method.

The two-dimensional Western blots indicated that post-translational modifications of cytoplasmic p40AUF1 were altered following TPA treatment. However, since ARE-binding complexes immunoreactive with anti-AUF1 antibodies were detected in the cytoplasm of both control and TPA-treated THP-1 cells (Fig. 3B), such modifications are unlikely to function by completely abrogating the ARE-binding activity of p40AUF1. Given that the focus of this study was to identify mechanisms contributing to the regulation of ARE-directed mRNA turnover in the THP-1 model system, the next question was thus to determine how RNA-bound (i.e. polysome-associated) AUF1 was modified before and after TPA treatment of this cell line. The loss of an acidic p40AUF1 variant without a significant change in the apparent molecular weight of the protein is consistent with a net loss of phosphate from p40AUF1 following TPA treatment. Accordingly, post-translational modifications of polysome-associated p40AUF1 were identified using

![Image](102x423 to 520x737)

**Fig. 2. Rapid mRNA stabilization contributes to accumulation of IL-1β and TNFα mRNAs in TPA-treated THP-1 cells.** The decay kinetics of IL-1β (A) and TNFα (B) mRNAs were evaluated in THP-1 cells treated with vehicle alone (0.1% Me2SO, 1 h) or TPA (10 nM, 1 h) by actD time course assay as described under "Experimental Procedures." Relative levels of IL-1β, TNFα, and β-actin mRNAs were quantified by RPA. The low abundance of IL-1β and TNFα mRNAs in unstimulated THP-1 cells necessitated much darker exposures for RPA gels than those displayed in Fig. 1. C, semilogarithmic plots of IL-1β (left) and TNFα (right) mRNA turnover, measured in control THP-1 cells (circles) or in cells treated with TPA (10 nM) for 1 h (triangles). Lines indicate first-order regression solutions for each time course. For the decay of TNFα mRNA in untreated THP-1 cells, three independent experiments were resolved simultaneously due to the extremely rapid disappearance of this mRNA following actD treatment.

### TABLE I

| mRNA  | 10 nM TPA | k ± S.E. | \( t_{1/2} \) | p versus untreated |
|-------|-----------|----------|---------------|-------------------|
| IL-1β |           |          |               |                   |
| 0     | -1.4 ± 0.2| 29       | <0.001        |                   |
| 1     | -0.23 ± 0.06| 150     | <0.004        |                   |
| 2     | -0.12 ± 0.02| 320     | <0.002        |                   |
| 4     | -0.114 ± 0.008| 365   | <0.001        |                   |
| TNFα  |           |          |               |                   |
| 0     | -5.3 ± 0.3 | 8        |               |                   |
| 1     | -0.7 ± 0.1 | 60       | <0.0004       |                   |
| 2     | -0.65 ± 0.07| 64      | <0.0001       |                   |
| 4     | -0.8 ± 0.1 | 54       | <0.0002       |                   |
MALDI-TOF mass spectrometry. MALDI-TOF analyses of polysome-associated p40\text{AUF1} purified from control versus TPA-treated THP-1 cells gave relative molecular weights (\(M_r\)) of 32,976 and 32,814, respectively (Fig. 5). The difference of 162 Da between these values is consistent with the loss of two phosphate groups from polysome-associated p40\text{AUF1} following TPA treatment. Furthermore, the determined \(M_r\) of p40\text{AUF1} purified from TPA-treated cells (32,814) was not significantly different from 32,818, the predicted \(M_r\) of a completely unmodified p40\text{AUF1} protein. The unphosphorylated protein (\(M_r\) 32,814) was not detected in polysome-associated AUF1 purified from control cells (Fig. 5A), nor was the modified protein (\(M_r\) 32,976) identified in analyses of p40\text{AUF1} purified from polysomes of TPA-treated cells (Fig. 5B). Whereas detection of additional cytoplasmic p40\text{AUF1} variants by two-dimensional Western analyses and the MALDI-TOF data, indicates that polysome-associated p40\text{AUF1} proteins do not significantly associate with mRNA. This finding, taken together with the two-dimensional Western analyses and the MALDI-TOF data, indicates that polysome-associated p40\text{AUF1} (i) is post-translationally modified in untreated THP-1 cells, most likely involving two phosphorylation events, (ii) loses these modifications following TPA treatment of THP-1 cells, and (iii) apparently lacks other post-translational modifications in this cell line.

Polysome-associated p40\text{AUF1} is Phosphorylated on Ser\text{\textsuperscript{83}} and Ser\text{\textsuperscript{87}} in Untreated THP-1 Cells—Since the preceding experiments strongly suggested the presence of two phosphate groups on polysome-associated p40\text{AUF1} in untreated THP-1 cells and their loss following TPA treatment, the next objectives were to verify the identity of the p40\text{AUF1} post-translational modifications as phosphates and to map their location(s) within the protein. To this end, polysome-associated p40\text{AUF1} purified from untreated THP-1 cells was digested with trypsin. Phosphorylated peptide fragments were then selected by IMAC across a Ga\textsuperscript{3+}-charged resin. MALDI-TOF analyses of Ga\textsuperscript{3+}-selected tryptic fragments indicated the presence of two phosphopeptides (Fig. 6A). The larger fragment (m/z 1539) was consistent with a single phosphate linked to peptide NEEDECHNPSRR, spanning amino acid residues 73–85 of p40\text{AUF1}. The smaller fragment (m/z 1051) was consistent with monophosphorylated HSEAATAQR, spanning residues 86–94. Single phosphate groups were conclusively indicated on each peptide by treatment with alkaline phosphatase, which caused a decrease of \(-80\) Da in the mass of each fragment (Fig. 6B). Since multiple potential sites of phosphorylation are present within each of these peptide fragments, specific modification sites were identified by limited carboxypeptidase Y digestion (Fig. 6C). In the larger phosphopeptide fragment (m/z 1539), phosphoserine was exclusively released as a Ser-Pro dipeptide (m/z 1381–1110), indicating that Ser\text{\textsuperscript{83}} was the site of phosphorylation in this fragment. Following digestion of the smaller fragment (m/z 1051) with carboxypeptidase Y, phosphoserine was retained in a His-Ser\textsubscript{87}-Glu-Ala tetrapeptide (m/z 516), permitting assignment of a single phosphate to Ser\textsuperscript{87} in this fragment. Based on these observations, we conclude that polysome-associated p40\text{AUF1} is phosphorylated on Ser\textsuperscript{83} and Ser\textsuperscript{87} in untreated THP-1 cells.

![Figure 3](image-url)  
**Fig. 3.** TPA treatment alters the distribution of cytoplasmic ARE-binding complexes containing AUF1. A, sequences of the AREs from human IL-1\(\beta\) and TNF\(\alpha\) mRNAs. AUUUA and GUUUG motifs are underlined. The sequence R\(\beta\) corresponds to a fragment of the coding sequence from rabbit \(\beta\)-globin mRNA. B, cytoplasmic activities interacting with the TNF\(\alpha\) ARE are detected by gel mobility shift assay. A \(^{32}\)P-labeled TNF\(\alpha\) ARE RNA substrate (0.2 nm) was incubated with crude cytoplasmic fractions (20 \(\mu\)g of protein) from THP-1 cells that were untreated (lanes 2–4) or treated for 1 h with 10 nm TPA (lanes 6–8). For a separate gel, comparable reactions were assembled using a \(^{32}\)P-labeled R\(\beta\) RNA substrate (lanes 10 and 11). To identify ARE-binding components that contained AUF1, additional binding reactions were treated with anti-AUF1 antiserum (\(\alpha\)-AUF1; lanes 4 and 8) and compared with binding reactions containing preimmune serum (p.i.; lanes 3 and 7). The location of the \(\alpha\)-AUF1-specific supershift is indicated. Since the supershifted complexes did not significantly migrate into the gel (bottoms of sample wells are visible at the top of the supershift signal), quantitative detection of these complexes was not possible. A replicate experiment yielded similar results. C, electrophoretic bands corresponding to complexes I (open bars) and II (solid bars) from lanes 2–4 and 6–8 in B were quantified using ImageQuant version 5.2 software (Amersham Biosciences). Blank-subtracted band intensity values were normalized to Intensity(complex I + II) = 1 from lane 2.
treated THP-1 cells and that these phosphate groups are lost following TPA treatment. Furthermore, observations that TPA treatment of THP-1 cells concomitantly effects changes in phosphorylation of polysome-associated p40AUF1 (Figs. 4B and 5), alterations in the distribution of cytoplasmic ARE-binding activities containing AUF1 (Fig. 3B), and stabilization of mRNAs containing AREs (Fig. 2), suggests the presence of a mechanistic link between AUF1 phosphorylation and the regulation of ARE-directed mRNA turnover.

DISCUSSION
Mechanisms regulating ARE-directed mRNA turnover rates are of substantial interest, since their manipulation presents an attractive target for restricting the expression of gene products contributing to the development of cancer and inflammatory diseases (3). Whereas recent studies have identified some signaling pathways contributing to these regulatory events, few details are available regarding either their molecular targets or the biochemical basis of their function in modulating mRNA turnover rates.

In this study, we have used acute TPA treatment of THP-1 cells as a model system to investigate mechanisms regulating ARE-directed mRNA decay. In particular, we queried how TPA-activated signaling systems might be transduced directly through the ARE-binding proteins responsible for catabolic targeting of these mRNAs. Treatment of THP-1 cells with TPA induced rapid stabilization of ARE-containing transcripts, concomitant with alterations in the activity of cytoplasmic ARE-binding complexes containing AUF1, and loss of phosphate from Ser83 and Ser87 of the major cytoplasmic AUF1 isoform, p40AUF1. These serine residues are both encoded within exon 2 of the human AUF1 gene (Fig. 6D), a domain that is conserved among the AUF1 genes sequenced to date. In rodents, Ser83 is retained, and the conservative Ser→Thr substitution at position 87 maintains the potential for phosphorylation at this site. Alternative pre-mRNA splicing of exon 2 sequences distinguishes p37AUF1 from p40AUF1 (6), the two cytoplasmic AUF1 isoforms expressed in THP-1 cells (Fig. 4A). Accordingly, p37AUF1 cannot be phosphorylated at comparable positions, indicating that signal transduction pathways regulating cytoplasmic AUF1 function through these phosphorylation events are specific for p40AUF1.

Although polysome-associated p40AUF1 was modified exclu-
sively by phosphate in THP-1 cells, other data maintain the possibility for additional post-translational modifications. First, the two-dimensional Western analyses clearly displayed at least three p40AUF1 species in the cytoplasm of untreated THP-1 cells (Fig. 4B, left), yet only a single polysome-associated p40AUF1 variant was detected by mass spectrometry (Fig. 5A). However, since the p40AUF1 analyzed by mass spectrometry was purified based on (i) its polysomal localization in THP-1 cells and (ii) its ability to bind a poly(U) column, p40AUF1 variants with poor or highly dynamic RNA-binding properties would not be retained for analysis. Second, in HeLa cells, a portion of the AUF1 population is ubiquitinated (18), yet no evidence for such modification was detected in THP-1 cytoplasm by two-dimensional Western blot or mass spectrometry. In the THP-1 system, however, it is conceivable that ubiquitinated AUF1 might not be detected if it accumulated in nuclei or the perinuclear space and was thus excluded from the soluble or polysomal cytoplasmic fractions or if ubiquitinated forms of

**Fig. 6.** Ser\(^{83}\) and Ser\(^{87}\) are phosphorylated in polysome-associated p40AUF1 from untreated THP-1 cells. A, tryptic fragments of polysome-associated p40AUF1 purified from untreated THP-1 cells were selected for binding to Ga\(^{3+}\) using IMAC and then analyzed by MALDI-TOF. \(M_r\) values corresponding to the principal Ga\(^{3+}\)-binding peptide fragments are indicated. Enhanced background signal was due to data collection in reflector positive mode, selected for its improved resolution of fragment mass. B, Ga\(^{3+}\)-binding tryptic peptide fragments of p40AUF1 were partially dephosphorylated using alkaline phosphatase as described under "Experimental Procedures" and then analyzed by MALDI-TOF. The arrows indicate ~80-Da, phosphatase-dependent shifts in fragment mass, consistent with the loss of a single phosphate group from each peptide. C, limited carboxypeptidase Y digests of Ga\(^{3+}\)-binding tryptic peptide fragments of p40AUF1 were analyzed by MALDI-TOF. \(M_r\) values correspond to peptide digestion products. Amino acid residues excised from each peptide were identified based on the resulting shift in \(M_r\) and are denoted by the schematics at the top. D, localization of p40AUF1 phosphorylation sites relative to its domain structure. Amino acid residues encoded by exon 2 (shaded) distinguish p40AUF1 from p37AUF1 and are located immediately N-terminal of the first RNA-binding domain (RRM1). Both sites of phosphorylation are contained within this alternatively expressed region of the protein. Alignment of homologous peptide sequences from mouse (GenBank\textsuperscript{TM} accession number I49070) and rat (GenBank\textsuperscript{TM} accession number BAB03467) indicate that the potential for phosphorylation at these sites is conserved (boxed), with Ser\(^{83}\) absolutely conserved, and a Thr residue substituted for Ser\(^{87}\) in rodent p40AUF1.
AUFI do not accumulate to significant levels in THP-1 cells due to rapid degradation by the proteasome. In any case, the potential exists for additional post-translational modifications of AUFI, which will be addressed in later studies.

Whereas cytoplasmic AUFI proteins are involved in the regulation of ARE-directed mRNA turnover, nuclear roles for AUFI have also been described, including the regulation of transcription (56–58). Sequences encoded by exon 2 are important for transactivation through p40AUFI, although this activity may also be regulated through selected signal transduction systems (56, 59). Recently, the potential for phosphorylation of p40AUFI at Ser96 and Ser97 was independently described based on in vitro kinase assays, which indicated that Ser97 was phosphorylated by protein kinase A, whereas Ser96 could be phosphorylated by glycogen synthase kinase-3β, although this was dependent on prior phosphorylation of Ser97 (60). Expression of Ser→Ala and Ser→Asp mutants of p40AUFI suggested that phosphorylation of Ser97 enhances the transactivation potential of the protein but that subsequent phosphorylation of Ser96 inhibits this function. Whereas our study confirms that Ser96 and Ser97 are targets for phosphorylation on p40AUFI, we furthermore provide the first evidence that these residues are phosphorylated in cells and link the loss of these phosphate groups with alterations in the activity of cytoplasmic ARE-binding complexes and concomitant inhibition of ARE-directed mRNA turnover.

ARE-directed mRNA decay was significantly slowed by TPA treatment of THP-1 cells concomitant with the loss of phosphate from p40AUFI. However, it is important to note that ARE-containing transcripts were still degraded relatively quickly. For example, TNFα mRNA was not stabilized beyond a half-life of about 1 h (Table I), although no phosphorylated p40AUFI was detected in the polysomal population at this point (Fig. 5B). This observation argues that multiple, partially redundant ARE-directed mRNA decay mechanisms may operate concurrently in this system, some of which are sensitive to TPA, whereas others remain active. For example, the other cytoplasmic AUFI isoform, p37AUFI, shows high affinity for ARE substrates (54) yet does not contain residues homologous to Ser96 and Ser97 of p40AUFI. The presence of distinct trans-acting factors is also supported by the population of cytoplasmic ARE-binding activities in TPA-treated THP-1 cells, some of which were not recognized by anti-AUFI1 antiserum (Fig. 3B). If these ARE-binding activities represent additional protein factors (like TTP, HuR, etc.), this supports a model for global regulation of ARE-directed mRNA decay mediated by the interplay of diverse factors operating in a combinatorial or mutually exclusive manner, thus greatly enhancing the sophistication of cellular control over the decay of individual mRNAs. For the TNFα mRNA, recent evidence also indicates the presence of an additional destabilizing element downstream of the ARE (61), which probably presents distinct protein binding preferences. Together, such integrated control mechanisms could be finely tuned by the activity of many different signaling systems yet define selectivity for specific mRNAs based on relative trans-factor binding affinity. Further support for this model is given by emerging examples of post-translational mechanisms influencing the activity or availability of individual ARE-binding proteins for interaction with cytoplasmic mRNA. For example, phosphorylation of TTP by p38MAPK correlates with rapid ARE-directed mRNA decay, possibly by enhancing the RNA-binding activity of TTP (62). However, an independent study indicates that TTP phosphorylation may be mediated by the p38MAPK-activated protein kinase 2 rather than p38MAPK itself (63). In any case, interactions between 14-3-3 proteins and TTP are linked to the phosphorylation status of TTP (64), which may influence its nucleo-cytoplasmic distribution. Recent work also suggests that p38MAPK-activated protein kinase 2 phosphorylation of heterogeneous nuclear ribonucleoprotein A0 may influence its interaction with cytokine mRNAs (65). The activity of the AMP-dependent protein kinase contributes to nuclear retention of HuR, thus restricting the ability of HuR to stabilize ARE-containing transcripts in the cytoplasm (66). In addition, HuR may be methylated in macrophages in response to lipopolysaccharide treatment (67). At present, the biochemical consequences of these post-translational modifications remain poorly defined. However, these examples illustrate how the activity or availability of selected ARE-binding factors may be modulated in response to diverse stimuli, thus altering the cytoplasmic population of ARE-binding proteins available to interact with, and hence direct the catalytic fate of, their mRNA substrates.

Accordingly, a major question concerns the biochemical significance of phosphorylation at Ser96 and Ser97 of p40AUFI. Since these phosphorylation events alter neither the abundance nor the nucleo-cytoplasmic distribution of this protein (Fig. 3A), we are interested in how these post-translational modifications influence the interaction of p40AUFI with its RNA substrates or other cellular factors. The localization of these phosphorylation sites immediately adjacent to the RNA-binding domains of p40AUFI (Fig. 6D) prompts the hypothesis that they regulate some feature(s) of its ARE binding activity, possibly by altering local charge distribution or by inducing conformational changes in the protein. In an accompanying article (68), we describe how phosphorylation at Ser96 and Ser97 modulates both (i) the RNA-binding thermodynamics of p40AUFI and (ii) the influence of this binding event on the conformation of ribonucleoproteins containing this protein.

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