Phosphorylation of p40AUF1 Regulates Binding to A + U-rich mRNA-destabilizing Elements and Protein-induced Changes in Ribonucleoprotein Structure*

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Messenger RNA turnover directed by A + U-rich elements (AREs) involves selected ARE-binding proteins. Whereas several signaling systems may modulate ARE-directed mRNA decay and/or post-translationally modify specific trans-acting factors, it is unclear how these mechanisms are linked. In THP-1 monocytic leukemia cells, phorbol ester-induced stabilization of some mRNAs containing AREs was accompanied by dephosphorylation of Ser83 and Ser87 of polysome-associated p40AUF1. Here, we report that phosphorylation of p40AUF1 influences its ARE-binding affinity as well as the RNA conformational dynamics and global structure of the p40AUF1-ARE ribonucleoprotein complex. Most notably, association of unphosphorylated p40AUF1 induces a condensed RNA conformation upon ARE substrates. By contrast, phosphorylation of p40AUF1 at Ser83 and Ser87 inhibits this RNA structural transition. These data indicate that selective AUF1 phosphorylation may regulate ARE-directed mRNA turnover by remodeling local RNA structures, thus potentially altering the presentation of RNA and/or protein determinants involved in subsequent trans-factor recruitment.

The steady-state level of any mRNA population is a collective function of its synthetic and degradation rates. Eukaryotic mRNAs decay across a broad kinetic spectrum, discriminated largely by the presence of specific cis-acting stability determinants within each transcript (reviewed in Refs. 1 and 2). For many labile mammalian transcripts, rapid cytoplasmic mRNA turnover is directed by A + U-rich elements (AREs) contained within their 3′-untranslated regions (reviewed in Ref. 3).

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Transcription of RNA and/or protein determinants involved in RNA structures, thus potentially altering the presentation of mRNA is directed by A + U-rich elements (AREs) contained within their 3′-untranslated regions (reviewed in Ref. 3). AREs constitute a diverse population of mRNA sequences that may interact with a wide variety of cellular protein factors. Binding of some factors, including AUF1 and tristetraprolin, is associated with acceleration of mRNA decay (4–7), whereas factors like HuR protect ARE-containing transcripts from degradation (8, 9). Furthermore, many additional ARE-binding factors exist for which specific roles in mRNA metabolism have not been defined (reviewed in Ref. 10). These myriad options for trans-factor occupancy on ARE targets present opportunities for multifactorial regulation of mRNA decay kinetics through these elements, including discrimination of mRNA targets based on ARE sequence composition, and differential trans-factor activation or inhibition by specific signal transduction pathways. The latter possibility is further supported by recent findings that some ARE-binding proteins may be post-translationally modified in response to diverse stimuli. For example, phosphorylation of tristetraprolin by components of the p38 mitogen-activated protein kinase pathway may alter its ARE binding activity (11, 12) and/or subcellular distribution (13). For HuR, phosphorylation events involving the AMP-dependent protein kinase are implicated in nuclear retention of the protein (14), whereas lipopolysaccharide treatment induces methylation of HuR (15). Finally, AUF1 may be modified by both ubiquitination (16) and phosphorylation (17, 39). However, whereas some potential regulatory events have been linked to these modifications, it remains unclear how they modulate the functions of ARE-binding proteins at the biochemical level, which conceivably may include alterations in the ability of each protein to interact with RNA substrates or other cellular components.

In the monocytic leukemia cell line, THP-1, interleukin-1β and tumor necrosis factor α (TNFα) mRNAs are stabilized following treatment with phorbol esters. Stabilization of these mRNAs was accompanied by changes in the activity of cytoplasmic ARE-binding complexes containing AUF1 and loss of phosphatase from Ser83 and Ser87 of the predominant polysome-associated AUF1 isoform, p40AUF1 (39). Current evidence indicates that AUF1 selectively binds and oligomerizes on ARE substrates (18, 19) and has the potential to remodel local RNA structure (20). In addition, cytoplasmic AUF1 is present in a multisubunit complex (17) containing other factors involved in the regulation of mRNA decay and translation, including the translation initiation factor eIF4G, poly(A)-binding protein, the gel mobility shift assay; GSK3β, glycogen synthase kinase 3β; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight; PKA, protein kinase A; RNP, ribonucleoprotein; TNFα, tumor necrosis factor α.
heat shock proteins Hsp70 and Hsc70 (21), and lactate dehydrogenase (22). Together, these findings suggest that AUF1 oligomers may function by recruiting additional trans-acting factors to ARE-containing mRNAs. It follows, therefore, that changes in AUF1 phosphorylation may influence mRNA decay kinetics by a number of mechanisms, including alterations in ARE-binding affinity, oligomerization potential, ribonucleoprotein (RNP) conformation, and interaction with other cytoplasmic proteins. Unlike the examples involving tristetraprolin and HuR (13, 14), phosphorylation of p40AUF1 does not appear to influence its nucleocytoplasmic distribution, since (i) both phosphorylated and nonphosphorylated p40AUF1 proteins were recovered from THP-1 cell polysomes, and (ii) no significant changes in the levels of nuclear or cytoplasmic AUF1 proteins were detected following phorbol ester treatment of these cells (39).

In this study, we have biochemically examined the influence of p40AUF1 phosphorylation at Ser83 and Ser87 on its interaction with the ARE from TNFα mRNA. First, we show that recombinant His8-p40AUF1 may be specifically, quantitatively, and independently phosphorylated in vitro at Ser83 by glycogen synthase kinase 3β (GSK3β) and at Ser87 by protein kinase A (PKA). Second, we employed gel mobility shift assays (GMSAs) and measurements of fluorescence anisotropy to show that phosphorylated His8-p40AUF1 retains specific ARE-binding activity but that the affinity of the p40AUF1/ARE interaction and the flexibility of the protein-bound RNA substrate varies among the different phosphorylated forms of the protein. Finally, using fluorescence resonance energy transfer (FRET), we demonstrate that the global conformation of the ARE substrate is compacted following binding of unphosphorylated, Ser83- and Ser87-phosphorylated, or Ser87-phosphorylated His8-p40AUF1. By contrast, His8-p40AUF1 phosphorylated at both Ser83 and Ser87 does not significantly condense the conformation of associated ARE substrates. Together, these data constitute the first biochemical evidence that post-translational modification of p40AUF1 regulates its ARE-targeting role and provide a potential mechanism for inhibition of ARE-directed mRNA turnover concomitant with loss of phosphate from polysome-associated p40AUF1.

EXPERIMENTAL PROCEDURES

RNA Oligonucleotides—RNA oligonucleotide substrates (see Fig. 3A) encoding the ARE from TNFα mRNA (TNFα ARE) or a portion of the rabbit β-globin mRNA coding sequence (Rb) were synthesized by Dharmacon Research (Lafayette, CO). FL-TNFα ARE contains a 5′-fluorescein (Fl) tag, whereas TNFα ARE-FI contains a 3′-linked Fl group. The RNA substrate Cy-TNFα ARE-FI contains 5′-cyanine 3 (Cy3) and 3′-Fl moieties. All RNA substrates were quantified spectrophotometrically as described (20). Where indicated, the TNFα ARE and Rb RNA substrates were 32P-labeled to specific activities of 3 × 105 cpm/μmol using γ-32P-ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (Promega, Madison, WI).

Preparation of Recombinant His8-p40AUF1—Plasmid pBAD/HisB-p40AUF1 was constructed by inserting the complete coding sequence of human p40AUF1 DNA into the polylinker of pBAD/HisB (Invitrogen) using standard subcloning techniques (23). The identity of the insert and continuity of the open reading frame were verified by automated DNA sequencing. Recombinant p40AUF1 was prepared as an N-terminal His8-fusion protein by arabinose induction of E. coli TOP10 cells transformed with pBAD/His-p40AUF1. His8-p40AUF1 was then purified by Ni2+-affinity chromatography and quantified by Coomassie Blue stained SDS-PAGE as described (19, 24), except that the loaded Ni2+ column was given an additional wash with 6 column volumes of Trition washing buffer (50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 20 mM imidazole, 5% polyethylene glycol 6000). Mock- or kinase-modified His8-p40AUF1 was then purified by Ni2+-affinity chromatography and quantified by Coomassie Blue-stained SDS-PAGE as described (19, 24), except that the loaded Ni2+ column was given an additional wash with 6 column volumes of Trition washing buffer (50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 20 mM imidazole, 1% Triton X-100) prior to His8-p40AUF1 elution to ensure complete removal of the kinases. Following purification, a sample from each preparation was analyzed by MALDI-TOF to verify quantitative phosphate transfer.

Mass Spectrometry—In-gel tryptic digestion, immobilized metal ion affinity chromatography, alkaline phosphatase reactions, carboxypeptidase Y digests, and detection of proteins and peptide fragments by MALDI-TOF mass spectrometry were all performed exactly as described previously (39). The apparent molecular weight of His8-p40AUF1 was calculated from the predicted amino acid sequence (GenBankTM accession number NM_002138 and pBAD/His vector system literature) (Invitrogen) using the AAStats program of the Biology Workbench version 3.2 (San Diego Supercomputer Center; available on the World Wide Web at www.workbench.sdsc.edu).

RNA-Protein Binding Assays—GMSAs using unmodified or phosphorylated His8-p40AUF1 and 5′-labeled RNA oligonucleotide substrates were performed essentially as described (18, 24), except that magnesium ions were not included in binding buffers, and heparin (5 μg/μl) and yeast tRNA (0.2 μg/μl) were included to compete for nonspecific RNA binding activities. Also, flanking regions of RNA substrates were not excised by nucleases prior to gel fractionation.

Fluorescence anisotropy was employed for all quantitative measurements of RNA-protein binding equilibria. Binding reactions containing the fluorescent RNA substrate FITC-TNFα ARE and varying concentrations of unmodified or phosphorylated His8-p40AUF1 were assembled in a final volume of 100 μl containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol, 0.5 mM EDTA, 0.1 μg/ml acetylated bovine serum albumin, and 1 μg/ml heparin. Following incubation at 25 °C for 1 min, anisotropy was measured using a Beamer 2000 variable temperature fluorescence polarization system (Panvera, Madison, WI) equipped with fluorescein excitation (490-nm) and emission (535-nm) filters. Preliminary on-rate experiments verified that anisotropic equilibrium was attained within 10–20 s at this temperature for all binding equilibria described herein (data not shown). For equilibrium binding experiments, the polarimeter was operated in static mode, with each sample scanned 10 times for 2 min each in freshly made 75 mM H3PO4 and air-dried. Filter-bound 32P was quantified by liquid scintillation counting. Non-specific binding of His8-p40AUF1 was determined by performing reactions in the absence of kinase. Substrate-linked 32P was then calculated as the difference in filter-retained 32P between reactions containing and lacking kinase. Additional reactions lacking the His8-p40AUF1 substrate indicated no significant retention of 32P via phosphorylation of the kinases themselves (data not shown). Phosphorylation reactions requiring both PKA and GSK3β were performed in tandem, with the PKA system allowed to proceed for 30 min prior to the addition of GSK3β and incubation for a further 60 min. For samples analyzed by mass spectrometry, γ-32P-ATP was omitted from reactions, and products were desalted and concentrated using ZipTipC18 columns (Millipore, Bedford, MA) according to the manufacturer’s instructions.

Large scale preparation of phosphorylated His8-p40AUF1 for biochemical analyses was performed similarly, but using 5 nmol of His8-p40AUF1 in a total volume of 300 μl PKA and/or GSK3β (50,000 units) were added as necessary, with samples incubated as above. Following phosphorylation reactions, samples were diluted 10-fold in Ni2+-affinity binding buffer (50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 20 mM imidazole, 5% polyethylene glycol 6000). Mock- or kinase-modified His8-p40AUF1 was then purified by Ni2+-affinity chromatography and quantified by Coomassie Blue-stained SDS-PAGE as described (19, 24), except that the loaded Ni2+ column was given an additional wash with 6 column volumes of Trition washing buffer (50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 20 mM imidazole, 1% Triton X-100) prior to His8-p40AUF1 elution to ensure complete removal of the kinases. Following purification, a sample from each preparation was analyzed by MALDI-TOF to verify quantitative phosphate transfer.

The total measured anisotropy (A0) of a mixture of fluorescent species exhibiting similar fluorescence quantum yields may be interpreted based on the intrinsic anisotropy and fractional concentration of each fluorescing species, given by A and f, respectively, using Equation 1 (25–27).
RNA Folding Regulated by AUF1 Phosphorylation

Kinase 3β in Vitro—In unstimulated THP-1 cells, polysem-associated p40AUF1 is phosphorylated on Ser87 and Ser83 (39). These modifications are lost following phorbol ester treatment of this cell line, concomitant with stabilization of some ARE-containing mRNAs and alterations in cytoplasmic ARE-binding activities containing AUF1. Three observations suggest that the effects of AUF1 phosphorylation on cytoplasmic mRNA turnover are likely to be manifested through p40AUF1. First, Ser83 and Ser87 are encoded by exon 2 of the AUF1 gene (32) and, due to alternative pre-mRNA splicing, are specific for the p45 and p40 isoforms of AUF1. Second, p45AUF1 is almost exclusively nuclear in THP-1 cells, whereas p40AUF1 is detected predominantly in the cytoplasm (39). Finally, p40AUF1 is the major polysem-associated AUF1 isoform in this cell line. Accordingly, the next objective was to determine how phosphorylation of p40AUF1 at Ser83 and Ser87 might alter its biological function. Given that these residues are located immediately upstream of the p40AUF1 RNA-binding domain (Fig. 1A), alterations in ARE-binding activity were postulated to be a possible consequence of these phosphorylation events.

In order to test this hypothesis, it was first necessary to generate purified, selectively phosphorylated forms of p40AUF1. Using the PhosphoBase version 2.0 database (33), PKA was predicted to specifically phosphorylate p40AUF1 at Ser87 (Fig. 1A). Furthermore, Ser83 presented a consensus phosphorylation site for GSK3, provided that Ser87 was previously phosphorylated. Recombinant, N-terminal His-tagged p40AUF1 was expressed in Escherichia coli TOP10 cells and purified by Ni2+-affinity chromatography to >95% purity as described under “Experimental Procedures” (Fig. 1B). In label transfer assays with [γ-32P]ATP, both GSK3β and PKA readily and independently phosphorylated the recombinant His8-p40AUF1 substrate, approaching a stoichiometry of 1 mol of phosphate/mol of protein in each case (Fig. 1C). For both phosphorylation reactions, SDS-PAGE analysis indicated a single principal 32P-labeled product (Fig. 1D).

To further confirm His8-p40AUF1 as the phosphorylated substrate, kinase reactions were repeated using unlabeled ATP and then analyzed by MALDI-TOF. A mock phosphorylation reaction (i.e. containing no enzyme) yielded a major product with a Mr of 37,803 (Fig. 1E), close to 37,793, the calculated Mr of unmodified His8-p40AUF1. By contrast, products identified in PKA- or GSK3β-programmed reactions gave Mr values indicating increases of 80 and 79 Da, respectively, consistent with the addition of a single phosphate to His8-p40AUF1 by each kinase. Since unphosphorylated His8-p40AUF1 was not detected in either experiment, phosphorylation by each kinase was deemed to be quantitative. Finally, a reaction programmed sequentially with PKA and then GSK3β on a substrate product with Mr of 37,963. The 160-Da difference relative to the unmodified protein indicates the addition of two phosphates to His8-p40AUF1 in these reactions.

Having demonstrated that His8-p40AUF1 may be quantitatively phosphorylated by PKA and GSK3β, it was subsequently necessary to confirm the sites of modification on the substrate protein. To this end, PKA-, GSK3β-, and PKA + GSK3β-phosphorylated His8-p40AUF1 were digested with trypsin. Released phosphopeptide fragments were then purified by immobilized metal ion affinity chromatography across a Ga3+-charged matrix and analyzed by MALDI-TOF (Fig. 2A). PKA-phosphorylated His8-p40AUF1 yielded a single Ga3+-binding fragment (m/z 1051), consistent with a single phosphate linked to peptide HSEAATTQR, spanning residues 86–94 of p40AUF1. Phosphorylation with GSK3β yielded a distinct fragment (m/z 1539), characteristic of monophosphorylated peptide NEEDEGHNS–SVP, spanning p40AUF1 residues 73–85. From PKA + GSK3β-

For all equilibria described in this work, total fluorescence intensity did not vary significantly as a result of protein binding (data not shown), thus validating interpretation of anisotropy by Equation 1. A sequential dimer binding mechanism yielding a tetrameric protein-RNA complex is defined by two equilibrium constants, K1 and K2, and performs three potential fluorescent species, R, P2R, and P4R (see Fig. 4A). Under conditions of limiting RNA (i.e. [protein]total ≫ [protein]free), measured anisotropy is thus related to the concentration of protein dimer (P2) by Equation 2 (18).

\[ A_i = \frac{A_{ki} + A_{hi}K[P_i] + A_{hi}K[P_i]^2}{1 + K[P_i] + K[P_i]^2} \]

Anisotropy data sets were also analyzed using a binary binding model described by Equation 3, where a single protein dimer interacting with an RNA substrate yields a single equilibrium constant (K).

\[ A_i = \frac{A_{ki} + A_{hi}K[R]}{1 + K[R]} \]

Application of binding algorithms to A, versus [P], data sets was performed by nonlinear least squares regression using PRISM version 2.0 (GraphPad, San Diego, CA). The appropriateness of all mathematical models was monitored by the coefficient of determination (R2) and analysis of residual plot nonrandomness to detect any bias for data subsets (PRISM version 2.0). Where indicated, pairwise comparisons of sum-of-squares deviations between mathematical models were performed using the F test, whereas pairwise comparisons of binding or anisotropy parameters between experiments used the unpaired t test. In both cases, differences exhibiting p < 0.05 were considered significant. Protein off-rate experiments to measure the dynamics of RNA-protein complexes were performed as described previously (18, 28).

Measurement of RNA Folding by FRET—Interpretation of RNA conformation in the P,R and P,R complexes by FRET required comparison of the protein dependence of FRET efficiency (E/H9251/H9251) calculated as 55.7 between fluorophores were calculated using Equation 5, where r represents the scalar distances measured in the presence of the acceptor using Equation 4 (29, 30).

\[ F_{EX} = 1 - \frac{F_{0}}{F_{0} + R_{0}^4} \]

RESULTS

Recombinant p40AUF1 Is Quantitatively Phosphorylated at Ser83 by Protein Kinase A and at Ser83 by Glycogen Synthase
phosphorylated His6-p40AUFI, Ga3+-binding fragments corresponding to both the PKA- and GSK3β-modified tryptic peptides were identified. For each Ga3+-binding peptide, the presence of a single phosphorylated residue was further confirmed by alkaline phosphatase digestion, which produced an ~80-Da decrease in fragment mass (Fig. 2B). Finally, the specific phosphorylation sites within each modified fragment were determined by limited carboxypeptidase Y digest (Fig. 2C). For the PKA-phosphorylated peptide (m/z 1051), phosphoserine was exclusively retained within a His-SerP-Glu tripeptide fragment (m/z 444), thus confirming Ser87 as the sole residue modified by PKA phosphorylation. In the GSK3β-phosphorylated peptide (m/z 1539), phosphoserine was released within a SerP-Pro-Arg tripeptide (leaving m/z 1113), permitting assignment of the GSK3β-modified residue to Ser83.

Together, these experiments confirm that recombinant His6-

**Fig. 1. Phosphorylation of His6-p40AUFI by PKA and GSK3β in vitro.** A, domain structure of p40AUFI. The N-terminal dimerization and C-terminal glutamine-rich (Q-rich) domains are common to all AUFI isoforms. The conserved RNP-2 and RNP-1 motifs within each RNA recognition motif (RRM) are indicated by solid boxes. Sequences encoded by exon 2 distinguish p40AUFI from the other cytoplasmic isoform, p37AUFI, and are denoted by the shaded box. Residues that are phosphorylated in polysome-associated p40AUFI purified from unstimulated THP-1 cells are indicated, along with consensus phosphorylation target sites for PKA and GSK3β. B, purified His6-p40AUFI was fractionated by SDS-PAGE prior to (undigested) or following (enterokinase (EK)-digested) excision of the N-terminal His6 tag and additional amino acids by digestion with enterokinase. Proteins were detected by staining with Coomassie Brilliant Blue R-250. C, in vitro phosphorylation of His6-p40AUFI by PKA and GSK3β was measured by label transfer assay as described under “Experimental Procedures” and plotted as the molar ratio of phosphate/protein as a function of incubation time. D, products from His6-p40AUFI phosphorylation reactions containing the kinases indicated were fractionated by SDS-PAGE. 32P-labeled protein products were detected by autoradiography. The migration position of His6-p40AUFI is indicated by the arrowhead (right). E, His6-p40AUFI phosphorylation reactions programmed without (mock) or with the indicated kinases were analyzed by MALDI-TOF mass spectrometry. M r values for the predominant peptides are indicated.
p40$^{\text{AUF1}}$ may be specifically and quantitatively phosphorylated in vitro by PKA at Ser$^{87}$ and by GSK3$\beta$ at Ser$^{83}$. In contrast with the data base prediction and a previous report (34), we find that phosphorylation of His$^{6}$-p40$^{\text{AUF1}}$ at Ser$^{83}$ with GSK3$\beta$ does not require prior phosphate linkage at Ser$^{87}$ and have confirmed this finding by label transfer assay, SDS-PAGE, and MALDI-TOF analyses. Finally, since the in vitro phosphorylation sites concur with those identified on polysome-associated His$^{6}$-p40$^{\text{AUF1}}$ purified from THP-1 cells, selective phosphorylation of His$^{6}$-p40$^{\text{AUF1}}$ with PKA and/or GSK3$\beta$ will be a versatile system for biochemically dissecting the influence of p40$^{\text{AUF1}}$ phosphorylation on its interaction with its RNA substrates or other macromolecules.

**Recombinant p40$^{\text{AUF1}}$ Associates with an ARE Substrate by Sequential Dimer Binding.**—To determine whether phosphorylation of His$^{6}$-p40$^{\text{AUF1}}$ influenced its RNA binding activity, mock-, PKA (Ser$^{87}$)-, GSK3$\beta$ (Ser$^{83}$)-, and PKA + GSK3$\beta$ (Ser$^{83}$ + Ser$^{87}$)-phosphorylated His$^{6}$-p40$^{\text{AUF1}}$ proteins were first purified by Ni$^{2+}$-affinity chromatography. Binding to RNA substrates was then qualitatively analyzed by GMSA. In each case, His$^{6}$-p40$^{\text{AUF1}}$ bound to a substrate containing the core ARE from TNF$\alpha$ mRNA (TNF$\alpha$ ARE) (Fig. 3B, lanes 2–5). Substrate specificity was also preserved, since binding was not observed to a fragment of the $\beta$-globin mRNA coding sequence (R$\beta$) (Fig. 3B, lanes 7–10). In the ARE-binding reactions, an RNA–protein complex of intermediate mobility was detected (Fig. 3B, complex a), characteristic of the sequential dimer binding mechanism employed by His$^{6}$-p37$^{\text{AUF1}}$ to associate with RNA substrates (18, 19). However, significant differences in ARE-binding activity were noted for the slowest mobility complexes. With the mock-phosphorylated protein, most bound ARE substrate migrated as a single complex (Fig. 3B, complex a).
Fluorescent RNA substrate, total measured anisotropy (\(0.2 \text{nM}\)) were incubated with purified, mock-phosphorylated His6-p40AUF1 or His6-p40AUF1 phosphorylated with PKA (Ser83), GSK3\(\beta\) (Ser85), or both kinases (Ser83 plus Ser85). Final protein concentration was 300 nm dimer in each case. Reaction products were resolved by GMSA. The arrowheads labeled a, b, and c denote migration positions of the principal RNA-protein complexes.

However, ARE substrate bound to the phosphorylated forms of His6-p40AUF1 was largely distributed across a population of two complexes (Fig. 3B, complexes b and c). Whereas the ARE substrate binds His6-p40AUF1 regardless of phosphorylation status, the variations in complex mobility observed by GMSA suggest that the conformation and/or flexibility of the resulting His6-p40AUF1-ARE complexes may be influenced by phosphorylation of the protein components.

To address these possibilities, complex formation between an ARE substrate and mock- or kinase-modified forms of His6-p40AUF1 was quantitatively assessed under solution equilibrium conditions by fluorescence anisotropy. Protein binding to a 5'-Fl-conjugated RNA substrate increases the anisotropy of the fluorophore, due to an increase in the rotational correlation time of the RNA-protein complex relative to the RNA alone and a decrease in the flexibility of single-stranded RNA molecules following protein binding (18, 25, 35). Accordingly, as the concentration of His6-p40AUF1 increases under conditions of limiting fluorescent RNA substrate, total measured anisotropy (\(A_0\)) of the RNA substrate increases (Fig. 4B). The mathematical relationship between \(A_0\) and protein concentration is dependent on the binding model describing their association. In the case of His6-p37AUF1 binding to the 5'-Fl-labeled TNF\(\alpha\) ARE, the protein dependence of anisotropy is well described by a highly dynamic, sequential dimer binding model (Fig. 4A), resolving to a tetrameric AUF1-RNA complex (18, 19). The equations relating \(A_0\) and protein concentration for this model are described under “Experimental Procedures” and permit solution of both equilibrium constants (\(K_1\) and \(K_2\)) as well as intrinsic anisotropy values for the free, protein dimer-bound, and protein tetramer-bound RNAs (\(A_{0,\text{free}}, A_{0,\text{dimer}}, A_{0,\text{tetramer}}\) respectively).

The fluorescence anisotropy assays yielded three principal observations indicating that His6-p40AUF1 and His8-p37AUF1 bind AREs by similar mechanisms. First, binding of mock-phosphorylated His8-p40AUF1 to the Fl-TNF\(\alpha\) ARE substrate was well described by sequential dimer binding (Equation 2; Fig. 4B, solid line), further confirmed by the random distribution of residuals about the regression solution (Fig. 4B, bottom). Second, the sequential dimer binding model was significantly preferred \((p < 0.0001 \text{ by F test})\) over an algorithm describing a single protein/RNA interaction (Equation 3; Fig. 4B, dotted line), demonstrating that the simpler binding model is clearly inappropriate in this case. Finally, interactions between His8-p40AUF1 and His8-p37AUF1 have been characterized. In these cases, the resulting ARE complexes may be influenced by phosphorylation of the protein components.

**Fig. 3.** GMSA analysis of phosphorylated His8-p40AUF1 binding to the TNF\(\alpha\) ARE. A, sequences of RNA substrates containing the ARE from human TNF\(\alpha\) mRNA (TNF\(\alpha\) ARE) or a fragment of coding sequence from rabbit \(\beta\)-globin mRNA (\(R\beta\)). B, \(32\text{P}\)-labeled RNA substrates (0.2 nm) were incubated with purified, mock-phosphorylated His8-p40AUF1 or His8-p40AUF1 phosphorylated with PKA (Ser83), GSK3\(\beta\) (Ser85), or both kinases (Ser83 plus Ser85). Final protein concentration was 300 nm dimer in each case. Reaction products were resolved by GMSA. The arrowheads labeled a, b, and c denote migration positions of the principal RNA-protein complexes.

**Fig. 4.** Characterization of His8-p40AUF1 binding the TNF\(\alpha\) ARE by fluorescence anisotropy. A, the sequential dimer binding model of AUF1-ARE complex formation. By this model, an AUF1 protein dimer \((P_2)\) interacts with an ARE substrate \((R)\) to generate the \(P_2R\) complex, described by the equilibrium association constant \(K_1\). This complex, in turn, may associate with a subsequent protein dimer to yield the \(P_4R\) complex, described by \(K_2\). B, binding reactions containing the fluorescent RNA substrate Fl-TNF\(\alpha\) ARE were assembled across a titration of mock-phosphorylated His6-p40AUF1 as described under “Experimental Procedures.” Fluorescence anisotropy was measured for each reaction and plotted as a function of protein concentration. The anisotropy data set was resolved by nonlinear regression using Equation 2 (solid line), with a residual plot prepared by subtracting the regression-derived anisotropy solution \((A_{0,\text{calc}})\) from the experimentally observed values \((A_{0,\text{obs}})\) at each tested protein concentration (lower panel). An additional regression solution is indicated for a single-site binding model, solved using Equation 3 (dotted line).
p40\textsuperscript{AUF1} and the Fl-TNF\textsubscript{A} ARE substrate were highly dynamic, with off-rate experiments yielding complex dissociative half-times of <15 s (data not shown), similar to those measured with His\textsubscript{6}-p37\textsuperscript{AUF1}-ARE complexes (18). Together, the similarities in the ARE-binding activities of His\textsubscript{6}-p40\textsuperscript{AUF1} and His\textsubscript{6}-p37\textsuperscript{AUF1} support a common RNA-binding mechanism for these proteins. Furthermore, they validate the use of the fluorescence anisotropy system to analyze His\textsubscript{6}-p40\textsuperscript{AUF1}/ARE solution binding equilibria, thus permitting quantitative assessments of ARE binding affinity, protein oligomerization potential, and RNA dynamics in His\textsubscript{6}-p40\textsuperscript{AUF1}-ARE complexes.

Phosphorylation of Recombinant p40\textsuperscript{AUF1} Alters ARE-binding Affinity and RNA Dynamics—ARE binding of all phosphorylated forms of His\textsubscript{6}-p40\textsuperscript{AUF1} conformed to the sequential dimer binding model, based on random residual distribution and poor representation by the binary binding algorithm (p < 0.0001 by F test; data not shown). The intrinsic anisotropy and association equilibrium constants for each protein binding to the Fl-TNF\textsubscript{A} ARE substrate are listed in Table I. Comparing these parameters between the various phosphorylated forms of His\textsubscript{6}-p40\textsuperscript{AUF1} revealed significant distinctions in their ARE-binding characteristics. For Ser\textsuperscript{83}-phosphorylated His\textsubscript{6}-p40\textsuperscript{AUF1}, an ~2-fold increase in affinity at the second binding step (K\textsubscript{b}) was observed relative to mock-phosphorylated protein. This was accompanied by a significant decrease in the intrinsic anisotropy of both the dimer-bound (ΔI\textsubscript{p2R}) and tetramer-bound (ΔI\textsubscript{p4R}) ARE substrates. These decreases in intrinsic anisotropy indicate that the mobility of the TF\textsubscript{A} ARE 5'-end is enhanced when complexed with His\textsubscript{6}-p40\textsuperscript{AUF1} phosphorylated on Ser\textsuperscript{87} relative to complexes formed with the unphosphorylated protein. Furthermore, the highly dynamic nature of the His\textsubscript{6}-p40\textsuperscript{AUF1}/ARE equilibrium (described above) raises the possibility that changes in A\textsubscript{p2R} and K\textsubscript{b} are linked, since enhanced RNA flexibility in the Ser\textsuperscript{87}-phosphorylated P\textsubscript{2R} complex may kinetically improve opportunities for the second dimer-binding event.

Based on the equilibrium binding studies, phosphorylation at Ser\textsuperscript{83} also influenced the ARE-binding activity of His\textsubscript{6}-p40\textsuperscript{AUF1}, although in a manner quite different from Ser\textsuperscript{87} phosphorylation. First, phosphorylation of His\textsubscript{6}-p40\textsuperscript{AUF1} at Ser\textsuperscript{83} did not detectably alter RNA flexibility in His\textsubscript{6}-p40\textsuperscript{AUF1}-ARE complexes relative to the mock-phosphorylated protein (based on A\textsubscript{p2R} and A\textsubscript{p4R}). Second, binding of the initial protein dimer to the ARE was inhibited by ~40% (K\textsubscript{b}) when phosphorylated at Ser\textsuperscript{83}. Third, comparison of ARE-binding parameters between the doubly phosphorylated protein and each singly phosphorylated species indicates that phosphorylation at Ser\textsuperscript{83} dominates the effects of Ser\textsuperscript{87} phosphorylation. This assertion is supported by (i) restriction of RNA mobility in the P\textsubscript{2R} (ΔI\textsubscript{p2R}) and P\textsubscript{4R} (ΔI\textsubscript{p4R}) complexes by phosphorylation at Ser\textsuperscript{83} plus Ser\textsuperscript{85}, relative to Ser\textsuperscript{87} alone, (ii) diminution of P\textsubscript{2R} affinity for a second His\textsubscript{6}-p40\textsuperscript{AUF1} dimer (K\textsubscript{b}) when phosphorylated at Ser\textsuperscript{83} plus Ser\textsuperscript{87} relative to Ser\textsuperscript{87}, and (iii) maintenance of Ser\textsuperscript{85}-mediated inhibition of the initial protein dimer-binding step (K\textsubscript{b}), regardless of Ser\textsuperscript{87} phosphorylation. Taken together, these data indicate that phosphorylation of His\textsubscript{6}-p40\textsuperscript{AUF1} induces several changes in its interactions with RNA substrates, including influences on the binding affinity of the initial protein dimer (Ser\textsuperscript{83}), RNA flexibility in His\textsubscript{6}-p40\textsuperscript{AUF1}-ARE RNP complexes (Ser\textsuperscript{87}), and enhancement of subsequent protein dimer recruitment (Ser\textsuperscript{87}). In addition, the phosphospecific effects on p40\textsuperscript{AUF1} activity are not additive, since phosphorylation at Ser\textsuperscript{83} abrogates the influence of modification at Ser\textsuperscript{87}.

Table I

| p40\textsuperscript{AUF1} phosphorylation status | A\textsubscript{p2R} | A\textsubscript{p4R} | K\textsubscript{b} | K\textsubscript{b} |
|-----------------------------------------------|------------------|------------------|----------------|----------------|
| Mock                                          | 0.0332 ± 0.0007  | 0.068 ± 0.001    | 0.109 ± 0.003  | 8.7 ± 1        |
| Ser\textsuperscript{83}                       | 0.0324 ± 0.0004  | 0.057 ± 0.002    | 0.098 ± 0.001  | 15 ± 1         |
| Ser\textsuperscript{87}                       | 0.0332 ± 0.0006  | 0.069 ± 0.002    | 0.109 ± 0.002  | 6.6 ± 0.8      |
| Ser\textsuperscript{83} + Ser\textsuperscript{87} | 0.033 ± 0.001    | 0.069 ± 0.001    | 0.105 ± 0.003  | 6.7 ± 0.1      |

\(a\) Anisotropy of the Fl-TNF\textsubscript{A} ARE RNA substrate in the absence of protein (mean ± S.D.; n ≥ 3).

\(b\) Parameters derived from regression solutions of A\textsubscript{p2R} versus [P\textsubscript{2R}] plots using the sequential dimer binding model of AUF1 association with the Fl-TNF\textsubscript{A} ARE substrate (Equation 2). Each value represents the mean ± S.D. of three independent experiments, each containing a minimum of 50 binding reactions.

\(c\) Values significantly different (p < 0.05) from those corresponding to the mock-phosphorylated protein.
creases in E_{FRET} are shifted to higher protein concentrations. In the case of the Ser^{87}-phosphorylated protein, this may be coupled to the increased flexibility of the ARE in each of the RNP complexes (described above). By contrast, Ser^{83}-phosphorylated His_{8}p40^{AUFI} requires higher protein concentrations to achieve complex formation, due to the 40% decrease in ARE binding affinity relative to the mock-phosphorylated protein (Table I). The most notable distinction, however, was observed with His_{8}p40^{AUFI}-ARE complexes containing the Ser^{83} plus Ser^{87}-phosphorylated protein, for which little change in E_{FRET} was detected as a function of protein concentration. These data indicate that, unlike mock-phosphorylated or singly phosphorylated His_{8}p40^{AUFI}, the doubly phosphorylated protein does not induce significant structural condensation of associated RNA substrates. Rather, RNA substrates bound by the doubly phosphorylated His_{8}p40^{AUFI} are retained in a relatively elongated conformation. In this manner, selective phosphorylation of p40^{AUFI} at Ser^{83} and Ser^{87} may dramatically alter not only the thermodynamics of formation but also the overall structure of RNPs resulting from its association with its RNA substrates.

**FIG. 5.** Analyses of RNA folding in His_{8}p40^{AUFI}-ARE complexes by FRET. A, schematic of RNA folding measured by FRET. The RNA substrate is labeled with Cy3 at its 5’-end and Fl at the 3’-end. Both fluorophores are excited at 490 nm; however, only Fl produces significant quantum emission near 518 nm. As such, loss of energy from Fl to Cy3 by FRET is detected by a diminution of fluorescence emission at 518 nm as described under “Experimental Procedures.” Since E_{FRET} varies inversely with the 6th power of the interfluorophore distance (Equation 5), small changes in RNA conformation profoundly influence E_{FRET}. B, fluorescence emission spectra (λ_{max} = 490 nm) of the Cy-TNF ARE-Fl RNA substrate incubated with increasing concentrations of His_{8}p40^{AUFI} (0, 0.5, 2.5, 10, 50, and 250 nM dimer). C, the fractional concentrations of free RNA (solid circles), P_{2}R complexes (open circles), and P_{4}R complexes (solid triangles) are shown as a function of His_{8}p40^{AUFI} concentration for each phosphovariant of the protein. Product concentrations are based on values of E_{K_{1}} and K_{2} given in Table I, with the total RNA concentration set to 2 nM. D, E_{FRET} of the Cy-TNF ARE-Fl RNA substrate was monitored in solution as a function of His_{8}p40^{AUFI} concentration for each phosphovariant of the protein as described under “Experimental Procedures.” Replicate experiments yielded similar results.
with an ARE substrate in vitro, based on GMSAs and fluorescence-based assays. Given the similarities in complex migration by GMSA, and the confident resolution of anisotropy data by the sequential dimer binding model in both cases, we concluded that the ability of His_6-p40AUF1 to form oligomeric His_6- p40AUF1-ARE complexes was not abrogated by changes in its phosphorylation status. That both Ser_83- plus Ser_87-phosphorylated and unphosphorylated p40AUF1 proteins are competent for RNA binding is further supported by their recovery from polysomal fractions of untreated and phorbol ester-stimulated THP-1 cells, respectively (39). However, two significant differences were observed in the ARE binding activities of the Ser_83- plus Ser_87-phosphorylated versus unphosphorylated His_6- p40AUF1 (Fig. 6). First, the unphosphorylated protein exhibited a small but significant decrease in affinity for the ARE substrate relative to Ser_83- plus Ser_87-phosphorylated His_6- p40AUF1, although this was only evident for interactions between the initial protein dimer and the ARE substrate (K_1). More dramatic, however, was that the ARE substrate is retained in an extended conformation when complexed with the Ser_83- plus Ser_87-phosphorylated protein but is folded into a more compact structure by unmodified His_6- p40AUF1. Extrapolating to the phosphorylation status of p40AUF1 in THP-1 cells, these data suggest that the ability of p40AUF1 to promote rapid mRNA decay may be coupled to its ability to maintain associated RNA substrates in elongated conformations, based on (i) stabilization of some ARE-containing mRNAs in THP-1 cells concomitant with loss of phosphate from Ser_83 and Ser_87 of polysome-associated p40AUF1 following phorbol ester stimulation (39) and (ii) retention of elongated RNA substrate conformation in vitro solely when both Ser_83- and Ser_87- are phosphorylated (this work). Whereas phosphorylation of His_6- p40AUF1 separately at Ser_83 or Ser_87 also yielded alterations in RNA binding affinity (lowered K_s for Ser_83-phosphorylated protein; elevated K_s for Ser_87) and RNA dynamics in the protein-bound state (increased RNA flexibility when Ser_87 phosphorylated), in neither case was adoption of the condensed RNA conformation significantly prevented in the RNP complex (Fig. 5). At present, physiological conditions yielding significant proportions of p40AUF1 phosphorylated exclusively at Ser_83 or Ser_87 have not been described. However, enhancement of K_s by phosphorylation at Ser_87 and its subsequent inhibition by Ser_83 phosphorylation reflects the influences of similar modifications on the
DNA-binding activity of the cyclic AMP response element binding protein. Phosphorylation of this transcription factor by PKA at Ser119 induces a 2–3-fold improvement in binding affinity for its cognate DNA substrate in vitro, but this enhancement is prevented by subsequent phosphorylation at a proximal Ser residue by GSK3 (37).

The next question is to identify the mechanisms whereby phosphorylation of p40\textsuperscript{AUF1} alters its RNA binding characteristics. Whereas additional studies will be necessary to define the contributions of different contact points to the overall stability and conformation of the AUF1-ARE complex, details accrued thus far suggest some putative foci of interest. One possibility is that changes in protein conformation are induced by phosphorylation at these residues. Another, and perhaps more likely scenario is that the introduction of localized negative charges in a region contiguous with the upstream RNA recognition motif of p40\textsuperscript{AUF1} (Fig. 1A) may directly influence interactions between this region and RNA substrates. By this mechanism, phosphorylation may induce localized repulsion from the RNA phosphodiester backbone. This could account for the enhanced ARE mobility observed in complexes with His\textsubscript{5} p40\textsuperscript{AUF1} phosphorylated at Ser37. In this case, however, compensation must be made for the enthalpic penalty arising from the unfavorable ionic interactions, since no loss of binding affinity was observed. Conceivably, this may involve favorable changes in entropy resulting from enhanced RNA flexibility and/or introduction of constructive ionic interactions at different sites. Localized electrostatic repulsion may also account for the decrease in binding affinity observed following phosphorylation at Ser35 (K\textsubscript{i}). Finally, the presence of phosphate groups conjugated to both Ser35 and Ser37 may interfere with protein-RNA contacts essential for adoption of the condensed RNA structure, provided that protein determinants in this region contribute to local remodeling of RNA substrates.

To place the influence of p40\textsuperscript{AUF1} phosphorylation within a broader perspective, we may speculate on means by which differences in AUF1-ARE complex conformation or dynamics contribute to alterations in mRNA decay rates. Previous data indicate that AUF1 initiates ARE-directed mRNA turnover through the targeted assembly of a multisubunit, trans-acting complex, involving AUF1 oligomerization and maximization of complex surface area (reviewed in Ref. 28). It follows, therefore, that phosphospecific alterations in the architecture of the AUF1-ARE RNP complex may expose or obscure specific RNA and/or protein determinants involved in subsequent factor recruitment. Furthermore, the rapid dynamics of AUF1/ARE interactions would ensure that changes in p40\textsuperscript{AUF1} phosphorylation status were quickly reflected in the cytoplasmic population of AUF1-ARE RNPs. Taken together, the influence of p40\textsuperscript{AUF1} phosphorylation on the conformation of AUF1-ARE RNPs, coupled with the multiplicity of ancillary factors that may subsequently interact with these complexes, provides a plethora of downstream binding events that may be sensitive to differential phosphorylation of p40\textsuperscript{AUF1}. Finally, the observation that both phosphorylated and nonphosphorylated forms of p40\textsuperscript{AUF1} interact with polyosomes in cells (39) raises the possibility that these modifications may serve as a switching mechanism for p40\textsuperscript{AUF1}, converting it from an “mRNA-destabilizing” to an “mRNA-stabilizing” factor.

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