AUFI Binding Affinity to A+U-rich Elements Correlates with Rapid mRNA Degradation*

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Rapid degradation of many labile mRNAs is regulated in part by an A+U-rich element (ARE) in their 3'-untranslated regions. Extensive mutational analyses of various AREs have identified important components of the ARE, such as the nonamer motif UUAUUUAUUU, two copies of which serve as a potent mRNA destabilizer. To investigate the roles of trans-acting factors in ARE-directed mRNA degradation, we previously purified and molecularly cloned the RNA-binding protein AUFI and demonstrated that both cellular and recombinant AUFI bind specifically to AREs as shown by UV cross-linking assays in vitro. In the present work, we have examined the in vitro RNA-binding properties of AUFI using gel mobility shift assays with purified recombinant His6-AUFI. We find that ARE binding affinities of AUFI correlate with the potency of an ARE to direct degradation of a heterologous mRNA. These results support a role for AUFI in ARE-directed mRNA decay that is based upon its affinity for different AREs.

Control of mRNA stability is an important component of eukaryotic gene expression and involves cis-acting elements that can be found in the coding region and/or 3'UTRs of mRNAs (reviewed in Refs. 1–6). One type of cis-acting instability element is comprised of the AREs found in the 3'UTRs of many unstable mRNAs (reviewed in Ref. 7). Many ARE-containing mRNAs are degraded by a sequential pathway involving removal of the poly(A) tract followed by degradation of the mRNA body (8–10). In most cases the poly(A) tract is thought to protect the mRNA from ribonuclease attack so that its removal permits degradation of the mRNA body (reviewed in Ref. 11). While it has been known for almost a decade that AREs are important for mRNA instability (12–15), the mechanism(s) by which they mediate mRNA turnover is still unknown.

Despite the presence of AREs in many different mRNAs, there is no single evolutionarily conserved A+U-rich instability sequence. Typically AREs contain multiple copies of the pentanucleotide AUUAU, often in conjunction with one or more U-rich regions (14). In addition, transfection studies indicate that as the number of tandemly repeated AUUAU motifs is increased in a reporter mRNA, its instability increases. Likewise, two copies of the nonamer motif UUAUUUAUUU act as a more potent destabilizer than a single nonamer motif (16, 17). Together, these analyses suggest that potent destabilizing AREs have high affinity binding sites for a mRNA decay factor(s).

In order to investigate how AREs function in mRNA turnover, we utilized a cell-free mRNA decay system to identify proteins that may be relevant to ARE-directed mRNA decay (8, 18, 19). To this end, we previously reported the purification, molecular cloning, and characterization of the ARE-binding protein AUFI (20). Cellular AUFI purified from cytoplasmic extracts of K562 human erythroid leukemia cells consists of a 37- and a 40-kDa isoformal. Cloning of the 37-kDa isoformal, p37AUFI, revealed two nonidentical RNA recognition motifs (21) and a short glutamine-rich region in the predicted amino acid sequence. Cloning of murine cDNAs suggests that the 40-kDa isoform may also contain 19 additional amino acids N-terminal to RNA recognition motif 1 (22). Both cellular and recombinant p37AUFI (hereafter referred to as AUFI) bind the AREs present in the c-fos and c-myc proto-oncogene mRNAs and the granulocyte-macrophage colony-stimulating factor cytokine mRNA as shown by UV cross-linking essays in vitro.

The potential influence of AUFI on ARE-directed mRNA decay extends beyond the control of cytokine and proto-oncogene expression. However, many mRNAs encoding components of G protein-coupled receptors, such as β-adrenergic receptors (β-ARs), contain AREs. Moreover, receptor levels are frequently subject to regulatory control. For example, exposure of smooth muscle cells to agonist down-regulates β2-AR mRNA levels by inducing degradation of the mRNA (23). Similarly, agonist-mediated destabilization of the human β1-AR mRNA appears to be dependent upon an ARE (24), and for both the human β1-AR and hamster β2-AR mRNAs decay occurs concomitantly with an increase in the cytoplasmic levels of AUFI (25). Since both cellular and bacterially expressed AUFI bind the β2-AR ARE (25), the reciprocal relationship between the half-life of β2-AR mRNA and the abundance of AUFI suggests that the half-lives of ARE-containing mRNAs may be dependent upon ARE-specific RNA binding affinity of AUFI.

Here, we test the hypothesis that the binding affinity of AUFI for an ARE should reflect the potency of that ARE as a mRNA destabilizer. Using purified recombinant His6-AUFI fusion protein, we find a direct relationship between the apparent Kd for ARE binding by AUFI and the potency of the ARE to direct mRNA decay. These results support a role for AUFI in ARE-directed mRNA decay that is based upon its affinity for different AREs.
AUF1 ARE-binding Activity and mRNA Decay

MATERIALS AND METHODS

All enzymes and plasmid vectors were obtained from Promega Corp. (Madison, WI) unless otherwise noted. All plasmid constructions were confirmed by both restriction enzyme analyses and dye-sequencing with Sequenase (version 2.0, U.S. Biochemical Corp.). Expression and Purification of His6-AUF1 Fusion Protein—His6-AUF1 fusion protein was expressed in bacteria and purified as described by Pende et al. (23). The concentration of purified recombinant His6-AUF1 was estimated by comparison with dilutions of BSA using Coomassie-stained SDS-polyacrylamide gels.

Gel Filtration Analysis of His6-AUF1—A 1 × 19-cm column of Sephacryl S-300 (Pharmacia Biotech Inc.) was equilibrated in factor-binding buffer (10 mM Tris, pH 7.5, 5.5 mM magnesium acetate, 100 mM potassium acetate). Four micromolar purified recombinant His6-AUF1 in a final volume of 300 μl of factor-binding buffer was loaded onto the column; 60 300-μl fractions were collected. Twenty microliters of each fraction was assayed for His6-AUF1 by Western blot analysis using anti-AUF1 antisera, and 5 μl of each fraction was analyzed for RNA-binding activity by mobility shift assay with radiolabeled c-fos ARE. Similarly, 400 mM purified recombinant His6-AUF1 in 300 μl of factor-binding buffer was loaded onto the column, and 20 900-μl fractions were collected. Protein in these fractions was precipitated with 10% trichloroacetic acid, 20 μg/ml lysozyme by incubation on ice for 30 min and centrifugation at 4 °C for 30 min. Each precipitate was resuspended in 10 mM Tris (pH 7.5) and assayed for His6-AUF1 by Western blot. The void volume was determined using blue dextran.

Construction of Plasmids for In Vitro RNA Synthesis—The sequences Rβ+ AT × 1, Rβ+ AT × 2, Rα+ AT × 3, and Rβ+ AT × 5 were synthesized by polynucleotides incorporated into DNA templates for RNA substrates containing (ATTT)5 was removed, and a 5′-TCGACTAAAGCTAAATAAATAAAT-3′ was ligated. For synthesis of c-AUF1 ARE-binding Activity and mRNA Decay

Binding of His6-AUF1 to Synthetic AREs Containing Tandem Repeats of AUUU—We previously reported the purification, molecular cloning, and characterization of the ARE-binding protein AUF1. Our hypothesis is that the binding affinity of AUF1 for an ARE should reflect the potency of that ARE as a mRNA destabilizer. To address this hypothesis biochemically, we have examined the RNA-binding properties of AUF1. For this purpose we expressed a His6-AUF1 fusion protein in Escherichia coli and purified the recombinant protein, which has an apparent molecular mass of about 51 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1, left panel). To confirm that this polypeptide was the ARE-binding protein, a binding reaction containing radiolabeled c-fos ARE was prepared with UV light, digested with RNase A, fractionated by SDS-PAGE, and detected by autoradiography. The 51-kDa fusion protein is the major cross-linked species (arrow).

RESULTS

Binding of His6-AUF1 to Synthetic AREs Containing Tandem Repeats of AUUU—We previously reported the purification, molecular cloning, and characterization of the ARE-binding protein AUF1. Our hypothesis is that the binding affinity of AUF1 for an ARE should reflect the potency of that ARE as a mRNA destabilizer. To address this hypothesis biochemically, we have examined the RNA-binding properties of AUF1. For this purpose we expressed a His6-AUF1 fusion protein in Escherichia coli and purified the recombinant protein, which has an apparent molecular mass of about 51 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1, left panel). To confirm that this polypeptide was the ARE-binding protein, a binding reaction containing radiolabeled c-fos ARE was prepared with UV light, digested with RNase A, fractionated by SDS-PAGE, and detected by autoradiography. The 51-kDa fusion protein is the major cross-linked species (arrow).
Fig. 2. Sequences of RNA substrates used in mobility shift assays with His6-AUF1. With the exception of the c-myc ARE, each complete RNA substrate contained the last 80 nucleotides of the rabbit β-globin coding region including the UGA termination codon and begins with an EcoRI site just 3′ to the T3 or SP6 bacteriophage promoter in each construct. A, the RβΔAU substrate is depicted with the rabbit β-globin sequence, shown as an open box, followed by nucleotides immediately 3′ of the UGA termination codon. Also shown is the site of insertion of AUUA or AUUU repeats in the Rβ+AUUUA and Rβ+(AUUU)5 substrates, respectively. The AUUAUUUAUU nonamer motifs are underlined in these sequences. B, the c-fos substrates are depicted with the rabbit β-globin sequence described above (open box) followed immediately by either the c-fos ARE or the mutant ARE3 (hatched box). Nucleotide sequences of the c-fos ARE and the ARE3 mutant are shown. U-to-A substitutions in the c-fos ARE3 mutant are underlined. C, the sequence of the c-myc ARE is shown.

RNA-binding activity of His6-AUF1 was assayed by non-denaturing gel mobility shift assays using radiolabeled substrates containing either one AUUU motif or from two to five tandem repeats of AUUU. (See Fig. 2 for RNA sequences.) AUUA motifs are present in the AREs of most unstable proto-oncogene and cytokine mRNAs, and the potency of an AUUU-containing sequence to act as a mRNA destabilizer is proportional to the number of tandemly repeated AUUU motifs. The AUUAUUUAUU nonamer motifs are parallel to their potencies as mRNA destabilizers. The low affinity binding to the U32 substrate is also consistent with its inability to promote mRNA degradation.

Binding of His6-AUF1 to Authentic c-fos and c-myc ARE Sequences—Our results show that His6-AUF1 has a relatively low affinity for a substrate with two tandem AUUU motifs (Table I). The c-fos ARE has two tandem AUUU motifs and a single AUUAUUAUUA motif separated by 19 nucleotides, while the c-myc ARE has two AUUAUUAUUAUUA motifs separated by 25 nucleotides (see Fig. 2). However, the c-fos and c-myc AREs are very potent mRNA destabilizers (17, 28). We therefore examined binding of His6-AUF1 to RNA substrates containing these AREs using the mobility shift assay. A plot of free RNA concentration versus fusion protein concentration revealed an apparent $K_d$ of 7.8 ± 0.4 nM for the c-fos ARE (Rβ+fosARE; Fig. 5A). High affinity binding does not require intact AUUAUUAUUA motifs, since His6-AUF1 binds the ARE3 mutant c-fos substrate containing single U-to-A substitutions in each AUUAUUAUUAUUA motif with an apparent $K_d$ of 20 ± 4 nM (Rβ+ARE3; Fig. 5B). (The difference in binding affinity between the wild-type and mutant c-fos ARE is not statistically significant (p > 0.05).) Likewise, His6-AUF1 binds the c-myc ARE with an affinity ($K_d = 21 ± 3$ nM) similar to that for the c-fos ARE (Table I). (The difference in binding affinity between the c-myc and c-fos ARE is not statistically significant (p > 0.05).) We conclude that AUF1 binds authentic c-fos and c-myc AREs with high affinity even though these AREs lack
multiple tandem repeats of AUUU. Thus, AUF1 is capable of binding a number of different AREs with high affinity.

**DISCUSSION**

The importance of AREs for mRNA turnover was first realized in 1986 (12, 14), yet it is still unclear how AREs function in mRNA decay. We have utilized biochemical approaches to identify trans-acting factors that bind AREs in order to relate such RNA binding to mRNA degradation. We previously purified, characterized, and molecularly cloned the ARE-binding protein AUF1, and in the present study we have examined binding affinities of a His_{6}-AUF1 fusion protein for A+U-rich sequences with defined relative potencies as mRNA destabilizers. Here, by determining apparent $K_{d}$ values for His_{6}-AUF1 binding, we demonstrate that AUF1-ARE binding affinity is directly related to the potency with which an ARE destabilizes a heterologous mRNA (Table I). Additionally, the affinity of His_{6}-AUF1 for the most potent destabilizing AREs is within the average range ($10^{-9}$ M) of affinities exhibited by several other RNA-binding proteins that recognize specific sequences or
require a higher concentration of active AUF1 for binding; mRNAs with AREs bound with lower affinities by AUF1 might have very short half-lives. Likewise, structures (29).

Certain A+U-rich sequences are more potent mRNA destabilizers than others, suggesting that the potencies of destabilizers are proportional to the binding affinities of a cellular decay factor(s). For example, when placed in the context of a heterologous, normally stable mRNA, AUUUA and (AUUU)2 are relatively ineffective as destabilizing elements; (AUUU)3 has a modest destabilizing effect; (AUUU)4 increases the decay rate further; and (AUUU)5 is the most potent destabilizer of the five (16, 17, 26). In fact, (AUUU)5 increases the degradation rate of a reporter mRNA to about the same extent as does the c-fos ARE (17). Likewise, His6-AUF1 binds the c-fos ARE (K_d = 7.8 ± 0.4 nM) and the (AUUU)3 substrate (K_d = 19 ± 7 nM) with similar affinities. (The differences are not statistically significant (p > 0.05)). Statistical analyses were used to determine significant differences (i.e. p < 0.05) between K_d values for His6-AUF1 binding to various RNA substrates, and as a result the RNA sequences used in this study can be grouped into three general classes: (i) RNAs that are either not bound or bound with low affinity by AUF1 and are not mRNA destabilizers (β-globin, U32, and AUUUA); (ii) RNAs that are bound with gradually increasing, moderate affinities by AUF1 and have a gradually increasing, partial destabilizing effect (AUUU)4 < (AUUU)3 < (AUUU)2; and (iii) RNAs that are bound with the highest affinity by AUF1 and are potent mRNA destabilizers (c-fos and c-myc AREs and (AUUU)5). Based upon these ranges of AUF1 binding affinities for various RNA substrates (low, moderate, high) and the relationship of high affinity binding to mRNA decay, the affinity of AUF1 for a mRNA may dictate the rate at which it is degraded. Therefore, cellular AUF1 concentration may be one determinant of mRNA half-life. In this regard we found that by comparing Western blots of K562 cytoplasmic extracts with known amounts of purified recombinant p37AUF1 (the isoform used in these studies) that there are approximately 3.2 × 10^4 cytoplasmic molecules of p37AUF1/cell (data not shown). Assuming a diameter of 20 μm for K562 cells and 50% of the cell volume as cytoplasm (30), the concentration of p37AUF1 is approximately 25 nM. This value is comparable with the apparent K_d for binding to the c-myc ARE. Thus low cellular concentrations of active AUF1 may be sufficient for binding to a mRNA that contains a high affinity AUF1-binding site such as the c-myc and c-fos AREs. Based upon our results, such mRNAs should have very short half-lives. Likewise, mRNAs with AREs bound with lower affinities by AUF1 might require a higher concentration of active AUF1 for binding; these mRNAs should be degraded at a slower rate than those with high affinity binding sites. Thus, the availability of active AUF1 for ARE binding is a potential mechanism by which cells could control mRNA turnover rates and one in which the decay of multiple mRNAs could be differentially regulated by AUF1 concentration. Support for this hypothesis is the relationship between AUF1 levels and ARE-directed mRNA destabilization observed in DDT1-MF2 hamster smooth muscle cells treated with (-)-isoproterenol. In this case, (-)-isoproterenol induces an increase in cellular AUF1 protein and mRNA levels. This increase in turn correlates with a faster decay rate for β2-adrenergic receptor mRNA, which contains an AUF1 binding site(s) in the 3′-UTR (25).

Recently, two groups reported that the functional sequence within an ARE appears to be the nonamer sequence UUAUUUAUU (16, 17). One copy of the sequence UUAUUUAUU in the 3′-UTR of a normally stable mRNA can increase its degradation rate compared with the wild-type mRNA, while two copies of the nonamer motif act as a very potent mRNA destabilizer. As depicted in Fig. 2, (AUUU)4 contains one copy of the nonamer (underlined in Fig. 2); (AUUU)3 contains two overlapping copies; and (AUUU)2 contains two copies that overlap by a single nucleotide. Consistent with the potencies of two copies or one copy of the nonamer as destabilizers, binding affinity of His6-AUF1 for (AUUU)5 is 3-fold and 5-fold greater than the binding affinities for (AUUU)4 or (AUUU)3, respectively (Table I). Thus, AUF1 may function in part via recognition of the nonamer motif.

Despite the potential importance of the nonamer motif UUAUUUAUU in ARE-directed mRNA decay, it is important to note that not all AREs found in unstable mRNAs contain this motif. For example, the portion of the c-myc ARE that functions as a very potent mRNA destabilizer does not contain this motif (28). While the c-myc ARE does contain noncontiguous AUUUUA motifs, destabilizing AREs that contain no AUUUUA motifs have also been identified. In addition, the presence of one or more AUUUUA motifs in an ARE may not be sufficient for effective degradation.

**Table I**

| RNA             | Relative degradation rate | Apparent K_d for AUF1 binding |
|-----------------|---------------------------|------------------------------|
| c-fos ARE       | ++++                      | 7.8 ± 0.4 nM                 |
| c-myc ARE       | ++++                      | 21 ± 3 nM                    |
| (AUUU)2         | ++++                      | 19 ± 7 nM                    |
| (AUUU)3         | ++                        | 60 ± 20 nM                   |
| (AUUU)4         | +                         | 100 ± 50 nM                  |
| (AUUU)5         | +                         | 150 ± 30 nM                  |
| AUUUA           | +                         | >500 nM                       |
| U32             | +                         | >750 nM                       |

**Fig. 4.** Rβ+U32 is not a high affinity His6-AUF1 binding substrate. RNA substrates contained the last 80 nucleotides of the rabbit β-globin coding region alone (Rβ) or linked to U32 (Rβ+U32). Binding affinity of His6-AUF1 for the Rβ+U32 or Rβ substrates was determined by electrophoretic mobility shift assays as described under “Materials and Methods.” Representative plots of [RNA]_free (nM) versus [His6-AUF1] for Rβ+U32 (triangles) and Rβ (open circles) are shown. The apparent K_d for binding to Rβ+U32 was determined from three separate experiments to be >500 nM, which was the highest protein concentration used. No binding to Rβ was detected.
mRNA destabilization (28). Moreover, analysis of a c-fos ARE mutant, ARE3, with single U-to-A substitutions in all three AUUUA motifs showed that intact AUUUA motifs are not required for rapid mRNA deadenylation but are important for rapid degradation of the mRNA body (9). The apparent \( K_d \) for His\(_6\)-AUF1 binding to the c-fos ARE and the ARE3 mutant were not statistically different (7.8 ± 0.4 nM and 20 ± 4 nM, respectively; \( p > 0.05 \)). Likewise, His\(_6\)-AUF1 bound a mutant c-myc ARE with single U-to-A mutations in both AUUUA motifs with affinity similar to wild-type c-myc ARE (data not shown). Thus, intact AUUUA motifs are not required for high affinity binding of His\(_6\)-AUF1 to the c-fos and c-myc AREs. Therefore, although the study of AUUUA and AUUUUAUUU motifs has contributed greatly to understanding of ARE-directed mRNA decay, it is evident that these motifs may constitute only a subset of important motifs within various AREs.

In conclusion, our results suggest that the affinity of AUF1 for particular ARE sequences is related to their potency as mRNA destabilizers. Future experiments will utilize AUF1 as a tool to define multiple classes of AREs and to define specific nucleotide requirements for AUF1 binding by selection of high affinity binding substrates from combinatorial libraries of RNA sequences (e.g., SELEX; reviewed in Ref. 31).

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