Individual RNA Recognition Motifs of TIA-1 and TIAR Have Different RNA Binding Specificities*

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TIA-1 and TIAR are two closely related RNA recognition motif (RRM) proteins which possess three RRM-type RNA binding domains (RRMs 1, 2, and 3). Although both proteins have been implicated as effectors of apoptotic cell death, the specific functions of TIA-1 and TIAR are not known. We have performed in vitro selection/amplification from pools of random RNA sequences to identify RNAs to which TIA-1 and TIAR bind with high affinity. Both proteins selected RNAs containing one or several short stretches of uridylate residues suggesting that the two proteins have similar RNA binding specificities. Replacement of the uridylate stretch with one or several short stretches of cytidine residues eliminates the protein-RNA interaction. Mutational analysis indicates that, for both TIA-1 and TIAR, it is the second RNA binding domain (RRM 2) which mediates the specific binding to uridylate-rich RNAs. Although RRM 2 is both necessary and sufficient for this interaction, the affinity for the selected RNA (as determined by filter binding assays) does increase when the second domain of TIAR is expressed together with the first and third domains ($K_d = 2 \times 10^{-8}$ M) rather than alone ($K_d = 5 \times 10^{-8}$ M). Although RRM 3 (of either TIA-1 or TIAR) does not interact with the uridylate-rich sequences selected by the full-length proteins, it is a bona fide RNA binding domain capable of affinity-precipitating a population of cellular RNAs ranging in size from 0.5 to 5 kilobases. In contrast, RRM 1 does not affinity-precipitate cellular RNA. The inability of RRM 1 to interact with RNA may be due to the presence of negatively charged amino acids within the RNP 1 octamer.

RNA-binding proteins are involved in a variety of fundamental cellular processes including RNA splicing, polyadenylation, RNA transport, and translation. Specific RNA sequences with which these proteins interact have been identified in some cases, but for the majority of RNA-binding proteins, the RNA targets are unknown. TIA-1 and TIAR are two closely related members of the RNA recognition motif (RRM) family of RNA-binding proteins (1, 2). The RRM (also known as the RNP motif, the RNP consensus sequence, the RNP-80, and the consensus sequence RNA binding domain) consists of 80–90 amino acids containing two stretches of 8 and 6 highly conserved residues called RNP 1 and RNP 2, respectively (3–5). TIA-1 and TIAR both possess three amino-terminal RRM domains and a glutamine-rich carboxyl terminus. The RRM domains of TIA-1 and TIAR are very similar with 79% amino acid identity between the first domains, 89% amino acid identity between the second domains, and 91% amino acid identity between the third domains. The carboxyl termini of the two proteins, in contrast, are only 51% identical in amino acid sequence (2).

Several observations suggest that TIA-1 and TIAR are involved in signaling apoptotic cell death. The introduction of purified TIA-1 or TIAR into the cytoplasm of thymocytes permeabilized with digitonin results in fragmentation of genomic DNA into nuclease-sized oligomers (1, 2). TIAR is translocated from the nucleus to the cytoplasm in response to exogenous triggers of apoptosis (6), and TIA-1 is phosphorylated by a serine/threonine kinase that is activated during Fas-mediated apoptosis (7).

Although these findings implicate TIA-1 and TIAR in the apoptotic process, the specific functions of these proteins have not been determined. The existence of Drosophila (8, 9) and Caenorhabditis elegans (10) homologs, each of which shares 46% amino acid identity with human TIA-1 and TIAR, indicates that TIA-1 and TIAR are proteins which have been evolutionarily conserved. In order to understand the functions of these proteins, both in cells undergoing apoptosis and in healthy cells, it is necessary to identify RNAs with which they interact. As a first step toward identifying their RNA targets, we have determined the RNA binding specificities of both TIA-1 and TIAR by selection/amplification from pools of random RNA sequences (11). We have found that both of these proteins interact with RNAs containing short stretches of uridylicates, and that for both proteins it is the second RNA-binding domain which mediates this sequence specificity.

**EXPERIMENTAL PROCEDURES**

**In Vitro Selection from Pools of Random RNA Sequences—RNA selection was based on the SELEX method (11). The pool of RNA sequences was in vitro transcribed from an oligonucleotide library provided by Dr. Jack Keene (Duke University). The oligonucleotides consisted of 68 random bases flanked at the 5’ end by the T7 RNA polymerase promoter sequence and at the 3’ end by 27 bases of common sequence. The RNA transcribed from the library was incubated with Escherichia coli-derived rTIA-1 or rTIAR which had been immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech Inc.) using monoclonal antibodies reactive with TIA-1 (203) or TIAR (1H10). The RNA was added to the immobilized protein in 300 μl of binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 0.05% Nonidet P-40, 0.5 mM MgCl₂, 0.125 mg/ml bovine serum albumin, 40 units/ml RNasin). After incubation for 20 min at room temperature, the beads were washed five times with 1 ml of NT2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂).
The RNA for UV-cross-linking assays was—

**Ultraviolet (UV)-cross-linking Assays Using Selected RNA Sequences**—The RNA for UV-cross-linking assays was in vitro transcribed using SP6 RNA polymerase (Promega). The DNA templates for transcription were individual reverse transcription-PCR products from the final round of in vitro selection/amplification (denoted “selected sequences”) or individual PCR products obtained by amplifying the oligonucleotide library prior to selection for TIA-1 or TIAR binding (“random sequences”). Both the selected sequences and random sequences were cloned into the TA vector (Invitrogen) and sequenced. Transcripts were internally labeled by including [³²P]-32P[γGTP (DuPont NEN) in the in vitro transcription reactions according to the protocol provided by Promega. UV-cross-linking was performed by incubating recombinant protein (5 pmol) with 1 × 10⁶ cpm of [³²P]-labeled RNA in 20 μl total volume of binding buffer (125 mM NaCl, 25 mM KCl, 5 mM HEPES pH 7.6, 2 mM MgCl₂, 3.6% glycerol) at 30 °C for 15 min. The binding reaction was incubated on ice and irradiated with UV light (254 nm) for 1 min using a Stratalinker 2400 (Stratagene). Following the UV irradiation, reactions were placed on ice and irradiated with UV light for 10 min using a Stratalinker 2400 (Stratagene). Following the UV irradiation, reactions were placed on ice and irradiated with UV light for 10 min using a Stratalinker 2400 (Stratagene). Following the UV irradiation, reactions were placed on ice and irradiated with UV light.

**Selection/amplification from pools of random RNA sequences was carried out as described under “Experimental Procedures.” The sequences of 20 TIA-1-selected RNAs and 20 TIAR-selected RNAs are shown. The uridylate stretches are underlined.

**RESULTS**

The TIA-1 and TIAR Proteins Select Uridine-rich RNAs—Recombinant TIA-1 and TIAR were each immobilized on Protein A-Sepharose with a monoclonal antibody (2G9 for TIA-1 and 1H10 for TIAR) that reacts with a carboxyl-terminal epitope of the protein. The immobilized protein was incubated with a large molar excess of in vitro transcribed RNA containing a region of 68 bases of random sequence. RNAs which bound to the protein were purified and amplified by reverse transcription-polymerase chain reaction. The DNA templates encoding the selected RNAs were transcribed using T7 RNA polymerase, and the process was repeated for a total of 5 cycles. After the final cycle, the DNA products were divided into the TA vector for sequencing. Twenty clones selected by TIA-1
and twenty clones selected by TIA were sequenced (Fig. 1). Eighteen of the twenty TIA-1-selected sequences, and nineteen of the twenty TIAR-selected sequences contain one or, in most cases, several short stretches of uridylate residues ranging in length from three to eleven nucleotides.

TIA-1 and TIAR Interact Specifically by UV-cross-linking with the Selected RNAs—

To confirm that TIA-1 and TIAR bind to the RNAs selected by the random RNA selection procedure, recombinant protein was incubated with \( ^{32} \text{P-labeled RNA and UV-irradiated as described under "Experimental Procedures." Following RNase A treatment, cross-linked protein was visualized by autoradiography of 10% SDS-polyacrylamide gels. Four TIA-1-selected RNAs (lanes 1–4), four TIAR-selected RNAs (lanes 9–12), three randomly picked RNAs (lanes 5–7 and 13–15), or a mixture of 20 randomly picked RNAs (lanes 8 and 16) were used. The numbers above lanes 1–4 and 9–12 correspond to the RNA sequences depicted in Fig. 1. The sequences of the randomly picked RNAs (A, B, C, and Mix) are not depicted. B, purified GST-TIA-1 or GST-TIAR was incubated with \( ^{32} \text{P-labeled selected RNA 1-1 (lanes 1–9) or R-2 (lanes 10–18) in the presence of increasing amounts of unlabeled competitor RNA (selected RNA 1-1, selected RNA R-2, or the mixture of 20 RNAs picked randomly from the library) as indicated. UV-cross-linking and subsequent analysis was performed as in A. The unlabeled competitor RNA was present in 10-, 20-, 40-, or 60-fold molar excess compared with labeled RNA. C, purified GST-TIA-1 was incubated with \( ^{32} \text{P-labeled selected RNA 1-1 in the presence of increasing amounts of unlabeled competitor RNA (selected RNA 1-1, truncated selected RNA 1-1, or mutated/truncated selected RNA 1-1) as indicated. The unlabeled competitor RNA was present in 10-, 20-, or 60-fold molar excess compared with labeled RNA.}

The Second RNA Binding Domain of Both TIA-1 and TIAR Binds Specifically to the Selected RNA Sequences—

TIA-1 and TIAR each contain three RRM-type RNA-binding domains. In order to investigate the contributions of the individual domains to the overall RNA binding activity of the protein, constructs which express various combinations of the RRM domains fused to GST were made by PCR cloning (see Fig. 3). As shown in Fig. 4, RRM 2, when expressed by itself or together with RRM 1 and/or RRM 3, is able to interact by UV-cross-linking with the selected RNA sequence (i.e. the RNA sequence selected by the full-length protein). The first and third RNA-binding domains of

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**Fig. 2.** UV-cross-linking of TIA-1 and TIAR to the selected RNA sequences. A, purified GST-TIA-1 or GST-TIAR was incubated with \( ^{32} \text{P-labeled RNA and UV-irradiated as described under "Experimental Procedures." Following RNase A treatment, cross-linked protein was visualized by autoradiography of 10% SDS-polyacrylamide gels. Four TIA-1-selected RNAs (lanes 1–4), four TIAR-selected RNAs (lanes 9–12), three randomly picked RNAs (lanes 5–7 and 13–15), or a mixture of 20 randomly picked RNAs (lanes 8 and 16) were used. The numbers above lanes 1–4 and 9–12 correspond to the RNA sequences depicted in Fig. 1. The sequences of the randomly picked RNAs (A, B, C, and Mix) are not depicted. B, purified GST-TIA-1 or GST-TIAR was incubated with \( ^{32} \text{P-labeled selected RNA 1-1 (lanes 1–9) or R-2 (lanes 10–18) in the presence of increasing amounts of unlabeled competitor RNA (selected RNA 1-1, selected RNA R-2, or the mixture of 20 RNAs picked randomly from the library) as indicated. UV-cross-linking and subsequent analysis was performed as in A. The unlabeled competitor RNA was present in 10-, 20-, 40-, or 60-fold molar excess compared with labeled RNA. C, purified GST-TIA-1 was incubated with \( ^{32} \text{P-labeled selected RNA 1-1 in the presence of increasing amounts of unlabeled competitor RNA (selected RNA 1-1, truncated selected RNA 1-1, or mutated/truncated selected RNA 1-1) as indicated. The unlabeled competitor RNA was present in 10-, 20-, or 60-fold molar excess compared with labeled RNA.**
both TIA-1 and TIAR, on the other hand, when expressed by themselves, do not cross-link to the selected RNA sequence, suggesting that the second domain is required for this interaction. GST alone does not cross-link to the RNA (data not shown). The finding that the second RNA-binding domains of TIA-1 and TIAR interact with the selected RNA sequence but not with a mixture of randomly picked RNAs (Fig. 4) suggests that these second RNA-binding domains of TIA-1 and TIAR bind to RNA with the same sequence specificity as their full-length counterparts.

To assess the strength of the interaction between full-length TIA or TIAR RRM truncation mutants and the selected RNA sequence, nitrocellulose filter binding assays were performed. Fig. 5 shows the normalized binding curves for the full-length GST-TIAR protein and several of the RRM truncation mutants. The K_d values shown below the plot are equal to the protein concentrations at which 50% of RNA is bound.

Unlike RRM 2 and 3, RRM 1 does not interact in vitro with RNA.
RNA Binding Specificities of TIA-1 and TIAR

Cellular RNAs—In an effort to identify cellular RNAs with which TIA-1 and TIAR interact, recombinant TIA-1 and TIAR GST fusion proteins were incubated with total RNA which had been extracted from \(^{32}\)P-labeled Jurkat cells. Following affinity precipitation with glutathione-Sepharose, associated RNA was isolated by phenol/chloroform extraction, separated on a formaldehyde-agarose gel, and transferred to nitrocellulose. For both TIA-1 and TIAR, the associated RNA appears by agarose gel electrophoresis to consist of a population of RNAs ranging in size from 0.5 to 5 kilobases (see Fig. 6). RRM 2 and RRM 3 when expressed individually, also interact with a population of RNAs. Surprisingly, however, for both TIA-1 and TIAR, the first RNA-binding domain does not affinity-precipitate RNA. This apparent lack of RNA binding activity is not due to RNase contamination of the RRM 1 protein preparations since in mixing experiments neither TIA-1 nor TIAR RRM 1 inhibits the RNA binding by the wild-type protein or the other truncation mutants (data not shown). Electrophoresis of the affinity-precipitates through 5% polyacrylamide gels (to allow visualization of snRNAs and tRNA) also failed to show RNA associated with RRM 1 (data not shown).

DISCUSSION

We have employed in vitro selection/amplification from pools of random RNA sequences to identify those RNA sequences for which the TIA-1 and TIAR proteins have the highest affinity. After five rounds of selection, 90% of the RNA sequences selected by both proteins contain short stretches of uridylate residues ranging in length from 3 to 11 nucleotides. The sequences do not appear to have similarity with regard to the distance between the uridylate stretches, the regions flanking the uridylate stretches, or the predicted secondary structure. UV-cross-linking experiments confirm that TIA-1 and TIAR bind the selected sequences, that the interaction is specific, and that the uridylate stretch is required for the interaction (see Fig. 2). The affinity of the interaction (K_d of 8 nM as determined by filter binding assays) is similar to that observed for other RNA-binding proteins (such as the hnRNP A1 protein) and their selected RNA sequences (15). Given the 80–90% amino acid identity between the RNA binding domains of TIA-1 and TIAR, it is not surprising that that the two proteins selected very similar RNA sequences.

Although TIA-1 and TIAR each contain three RRM-type RNA-binding domains, our results suggest that the second domain of each of these proteins mediates the sequence-specific binding. Mutational analysis demonstrates that RRM 2 is both necessary and sufficient for binding to the RNAs selected by the full-length protein. Studies of other RRM-type RNA-binding proteins (hnRNP C, U1A, and U1 70K), have suggested that their RNA binding specificities are dependent on amino acids immediately carboxyl-terminal to the RRM domain (13, 16, 17). Our results are not inconsistent with this, since the 15 amino acids linking the second and third RRMs are present in each mutant that contains RRM 2. A single RRM of a multi-RRM protein having the same sequence specificity as the full-length protein is not unique to the TIA-1 and TIAR proteins. The U1 snRNP A protein has two RRMs, only one of which appears to be required for binding to U1 RNA (18, 19). Similarly, the third RNA binding domain of Hel-N1, like the full-length protein, binds to the 3′ untranslated region of c-myc mRNA (20). An analysis of the binding specificity of the hnRNP A1 protein, on the other hand, shows that its two RRMs have sequence specificities which differ both from each other and from the full-length protein (15). Thus, within the RRM family of RNA-binding proteins, there does not appear to be uniformity with regard to the contribution of individual RRMs to overall binding specificity.

Although the third RNA-binding domains of TIA-1 and TIAR do not bind to the uridylate-rich RNAs selected by the full-length protein, they clearly do have RNA binding activity as illustrated by their ability to affinity-precipitate cellular RNA (see Fig. 6). The ability of RRM 3 to bind to cellular RNA but not to the mixture of random RNAs in vitro transcribed from the oligonucleotide library (see Fig. 4) may be due to differences in the sensitivities of the assays. It is quite possible that RRM 3 interacts with specific RNA sequences (that differ from the uridylate stretches) but that these sequences were not identified using the selection/amplification method because they are of low affinity or because, as has been demonstrated for U2 snRNP-B′ binding to U2 RNA, the interactions require additional proteins (21–23).

In contrast, we have been unable to demonstrate any RNA binding activity for the first RNA-binding domain of either TIA-1 or TIAR. The RNP 1 octamer of the first RNA-binding domains of TIA-1, TIAR, and homologous proteins found in Drosophila (8, 9) and C. elegans (10) are all somewhat atypical in that they contain aspartic acid in the first position (see Fig. 3 for human TIA-1 and TIAR RNP 1 sequences). The RNP 1 octamers of the second and third RNA-binding domains of the TIA proteins each have a lysine in the first position and thereby resemble the RNP 1 sequences of the majority of RRM-type proteins which contain positively charged amino acids (either arginine or lysine) at that position (5, 24). It has been proposed that the four-stranded antiparallel β-sheet within the RNA-binding domain of RRM-type proteins provides a general surface for RNA binding, and that the highly conserved basic and aromatic amino acids of RNP 1 and 2 (which form two of the β-strands) are involved in direct interactions with RNA (5, 13, 24, 25). The first residue of the RNP 1 octamer of the U1A RRM 1 has been shown to be critical for U1A binding to U1 RNA; replacement of the arginine with the uncharged glutamine completely abolishes binding to U1 RNA (26, 27). One other RRM-type protein with a negatively charged amino acid in the first position of RNP 1 is ASF/SF2. The ASF/SF2 RNP 2 RNP 1 octamer begins with an aspartic acid (although there appears to be debate as to whether the eight amino acid sequence beginning with aspartic acid, or an eight amino acid sequence three residues downstream and beginning with a glycine, is the actual RNP 1 sequence) (24, 28). Additional studies are necessary to determine whether the negatively charged aspartic acid
of TIA-1/R NRP 1 affects the RNA binding activity of RRM 1. It is possible that RRM 1 may preferentially interact with RNA that has been modified in ways which decrease its net negativity (e.g., by capping).

Uridylicate stretches, frequently found in regulatory regions of RNAs such as the 3’ splice site of introns (29, 30) and 5’ and 3’ untranslated regions (20, 31), appear to be common targets of RRM-type RNA-binding proteins. The hnRNP C and Hel-N1 proteins both selected sequences containing short uridine stretches when similar selection/amplification approaches were used with these proteins (13, 20), and the Sex- lethal (Sxl) protein of Drosophila has been shown to interact with poly(U) sequences in transformer (tra) and Sxl pre-mRNAs (32–36). Because cells contain a multitude of RNA-binding proteins, some of which may compete with TIA-1 and TIAR for binding to RNA, it is important to characterize the RNA binding activity of TIA-1 and TIAR not only in vitro but also in cells. The similarity between the uridylicate stretch consensus sequences identified for the TIA-1/R and hnRNP C proteins, together with the size distribution of the cellular RNAs interacting in vitro with TIA-1/R, suggested to us that the TIA proteins may associate in cells with pre-mRNA or mRNA. Although we have not been able to demonstrate an in vivo interaction between endogenous TIA-1 or TIAR and RNA, both of these proteins can be isolated by oligo(dT) affinity chromatography of lysates prepared from UV-irradiated TIA-1 or TIAR COS cell transfec- tants indicating that, at least when overexpressed, the proteins interact in cells with polyadenylated RNA (37, 38). The identification of uridylicate stretches as high affinity binding sites for TIA-1 and TIAR, together with the demonstration of in vivo interactions between these proteins and polyadenylated RNA, suggest that the TIA proteins might interact with regulatory regions of RNA transcripts to modulate gene expression. It is not known how the RNA binding activities of these proteins relate to their effector functions in apoptotic cell death. An intriguing possibility is that the TIA proteins regulate the processing or translation of RNA transcripts encoding mediators of apoptosis. Alternative splicing of ICE (39), Ich-1 (40), and bcl-x (41) can produce mRNAs encoding both positive and negative regulators of apoptosis. Studies aimed at determining whether the TIA proteins bind to uridine-rich regions of such RNAs are under way.

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