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Tristetraprolin controls the stability of *cIAP2* mRNA through binding to the 3′UTR of *cIAP2* mRNA

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ABSTRACT

cIAP2 is a key regulator of programmed cell death and the NF-κB pathway. Here, we investigated the post-transcriptional regulation of cIAP2 expression by tristetraprolin (TTP). Our results showed that overexpression of TTP reduced the stability of *cIAP2* mRNA and the expression level of cIAP2. In addition, TTP destabilized a luciferase mRNA containing *cIAP2* mRNA 3′UTR. *cIAP2* mRNA 3′UTR contains four AU-rich elements (AREs) and the 2nd ARE was responsible for the TTP-mediated destabilization of the *cIAP2* mRNA. RNA EMSA revealed that TTP directly bound to 42 nucleotides from the 3′UTR of *cIAP2* mRNA containing the 2nd ARE. However, the 42 nucleotides did not promote TTP-dependent destabilization of mRNA and did not recruit the decapping enzyme Dcp2 and the 5′–3′ exonuclease Xrn1. When we used a 52 nucleotide sequence containing an additional 5 nucleotides from *cIAP2* mRNA 3′UTR at both ends, this long nucleotide sequences recruited Dcp2 and Xrn1 and promoted TTP-dependent destabilization of mRNA. Collectively, our results suggest that TTP can bind to the 2nd ARE of *cIAP2* mRNA 3′UTR and destabilize *cIAP2* mRNA by forming complexes with Dcp2 and Xrn1. However, while a short nucleotide sequence containing the 2nd ARE of cIAP2 mRNA can recruit the TTP binding, this cannot recruit Dcp2 and Xrn1 and cannot induce TTP-mediated destabilize the mRNA. Instead, additional nucleotide sequences are required to recruit Dcp2 and Xrn1 and to destabilize mRNA.

1. Introduction

The inhibitors of apoptosis (IAP) proteins are a family of cell-death regulators that can block apoptosis induced by diverse stimuli through direct interaction with inducers and effectors of apoptosis [1,2]. The cIAP1 and cIAP2 proteins are ubiquitin ligases that were initially identified in association with tumor necrosis factor receptor 2 (TNFR2) [3]. Subsequent studies showed that cIAP1 and cIAP2 are recruited to TNFR1 through TRAF2, where they act as regulators of NF-κB [4]. In the canonical NF-κB pathway, cIAP1 and cIAP2 promote the K63 ubiquitination of RIP1 (receptor interacting protein 1) and activate the NF-κB-signaling pathway [5,6]. On the contrary, in the non-canonical NF-κB pathway, cIAP1 and cIAP2 induce the degradative K48 ubiquitination of NIK (NF-κB-inducing kinase) and block the NF-κB pathway [7,8].

The expression of cIAP1 and cIAP2 is tightly regulated both at the transcriptional and translational levels. Their expression is induced by TNF-α [9], protein kinase C (PKC) [10], Akt [11] and environmental stress [12]. Omi/HtrA2 and Smac/DIABLO regulate the stability of cIAP1 and cIAP2 [13,14], as well as autoubiquitylation and proteasomal degradation [15]. The highly regulated expression of cIAP proteins reflects their importance in controlling cell fate. It has been reported that overexpression or genetic mutation of cIAP1 and cIAP2 is associated with cancers, and may promote tumor cell survival [16–18].

Post-transcriptional regulation of gene expression can be mediated by AU-rich elements (AREs) located in the 3′ untranslated region (UTR) [19]. Many mRNAs with a short half-life contain AREs [20]. ARE binding proteins such as BRF1 and tristetraprolin (TTP) can recognize AREs and promote decay of the transcripts [21,22]. Previously, we reported that BRF1 can suppress the expression level of cIAP2 and increase the cisplatin-sensitivity of human head and neck cancer cells [23]. In the present study, we investigated the role of TTP in the regulation of *cIAP2* gene expression in human cancer cells. TTP promotes ARE-mediated mRNA decay by interacting with several protein components of P-bodies (processing bodies) including decapping enzyme Dcp2, 5′–3′, exonuclease Xrn1, and Ago [22,24]. Our data demonstrate that *cIAP2* mRNA contains four AREs in the 3′UTR and that TTP operates its destabilizing activity through direct binding to the second ARE of *cIAP2* mRNA.

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Interestingly, we found that the binding of TTP to the 3′UTR of *cIAP2* mRNA is not enough for degradation of the target gene. Besides the sequences for TTP binding, several additional sequences are required for recruiting Dcp2 and Xrn1 and for degradation of the target gene.

2. Materials and methods

2.1. Cells

293 EBNA and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM). Both cell lines were supplemented with 10% FBS (WELGENE, Daegu, Korea) and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Semi-quantitative RT-PCR

Three microgram of DNase I-treated total RNA was reverse transcribed using oligo-dT and M-MLV-reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed using EF-Taq polymerase (Solgent, Daejeon, Korea) and appropriate primers. PCR primer pairs were as follows: TTP: CGCTACAAGACTGAGCTAT, GAGGTAGA ACTTGTGACAGA; cIAP2: CCCTTTTCTTCCCCATTCAT, AAACCAGCAC GAGCAAGACT; GAPDH: ACATCAAGAAGGTGGTGAAG, CTGTTGCTG TAGCCAAATTC.

2.3. Plasmids, transfections and luciferase assay

The pcDNA6/V5-TTP plasmid has been described previously [25]. 293 EBNA cells or HeLa cells were transfected with pcDNA6/V5-TTP using the TurboFect™ *in vitro* transfection reagent (Fermentas, Hanover, MD).

A variety of deletion mutants of *cIAP2* 3′UTR were PCR amplified from the cDNA of 293 EBNA cells using EF-Taq polymerase (Solgent). PCR primer pairs were as follows: Frag-ARE-1–4: CTCG AGCTTTCATGAAGAAGAACC, GCGGCCGCAAGAGTAATTTTTGTTG; Frag-ARE-1: CTCGAGCTTTCATGAAGAAGAACC, GCGGCCGCAATTA GGATAAAAGT; Frag-ARE-2: CTCGAGCTTTCATGAAGAAGAACC, GC GGCCGCCAATGTTTTTTGAGTTGT; Frag-ARE-3: CTCGAGCTTT- CATG AAGAAGAACC, GCGGCCGCTACCTCTTTTTCGTTCAT. Underlined letters are restriction enzyme sites. PCR products were inserted into the *Xho*I/*Not*I sites of a psiCHECK2 luciferase expression vector (Promega). Seven oligonucleotides containing AUUUA motifs (ARE-1W, ARE-2W, ARE-3W, ARE-4W, ARE-2W-1, ARE-2W-2, and ARE-2W-3) of the *cIAP2* mRNA 3′UTR were synthesized at Integrated DNA Technologies (Coralville, IA) (Table 1). Mutant oligonucleotides in which AUUUA pentamers were substituted with AGCA were also synthesized. The oligonucleotides were ligated into the *Xho*I/*Not*I site of the psiCHECK2 vector.

For luciferase assays, 293 EBNA cells were co-transfected with various kinds of psiCHECK-cIAP2 3′UTR constructs and pcDNA6/V5-TTP using TurboFect™ *in vitro* transfection reagent (Fermentas). Transfected cells were lysed with lysis buffer (Promega) and mixed with luciferase assay reagent (Promega) and the chemiluminescent signal was measured in a SpectraMax L Microplate (Molecular Devices). Firefly luciferase was normalized to Renilla luciferase in each sample. All luciferase assays reported here represent at least three independent experiments, each consisting of three wells per transfection.

2.4. Electrophoretic mobility shift assay (EMSAs)

The biotinylated RNA probes for wild-type (cIAP2-ARE-2W, UUUGGUUUCCUUAAAAUUUUUAUUUAUUUACAACUCAAAAAACA-UUGUUUUG; cIAP2-ARE-2W-1, UUUCCUUAAAAUUUUUAUUU-AUUUACAACUCAAAAAACAUUG-) were synthesized by Samchully Pharm. Co. Ltd. (Seoul, Korea). A mutant RNA probe in which AUUUAUUUA sequences of cIAP-ARE-2W-1 were substituted with AGCAGCA was used as a negative control. Cytoplasmic extracts were prepared from pcDNA6/V5-TTP transfected 293 EBNA cells using NE-PER® Nuclear and Cytoplasmic extraction Reagent (Thermo Pierce Biotechnology Scientific, Rockford, IL). RNA EMSA was performed using the Lightshift® Chemoluminescent EMSA Kit

Table 1
Oligonucleotides used to analyze *cIAP2* mRNA 3′UTR.

Oligonucleotides	Sequences
Oligo-ARE-1W	F:TCGAGCTAAACTTTAGAATTA**ATTTA**TTAAATGTATTATAACTTTA
	R:GGCCGCTAAAGTTATAATACATTTAA**TAAAT**TAATTCTAAAGTTTAGC
Oligo-ARE-1M	F:TCGAGCTAAACTTTAGAATTA**AGCA**TTAAATGTATTATAACTTTA
	R:GGCCGCTAAAGTTATAATACATTTAA**TGCT**TAATTCTAAAGTTTAGC
Oligo-ARE-2W	F:TCGAGTTTGGTTTCCTTAAAATTTTT**ATTTATTTA**CAACTCAAAAAACATTGTTTTGGC
	R:GGCCGCCAAAACAATGTTTTTTGAGTTG**TAAATAAAT**AAAAATTTTAAGGAAACCAAAC
Oligo-ARE-2M	F:TCGAGTTTGGTTTCCTTAAAATTTTT**AGCAGCA**CAACTCAAAAAACATTGTTTTGGC
	R:GGCCGCCAAAACAATGTTTTTTGAGTTG**TGCATGCT**AAAAATTTTAAGGAAACCAAAC
Oligo-ARE-3W	F:TCGAGCATTGTTTTGTGTAACAT**ATTTA**TATATGTATCTAAACCATGC
	R:GGCCGCATGGTTTAGATACATATA**TAAAT**ATGTTACACAAAACAATGC
Oligo-ARE-3M	F:TCGAGCATTGTTTTGTGTAACAT**AGCA**TATATGTATCTAAACCATGC
	R:GGCCGCATGGTTTAGATACATATA**TGCT**ATGTTACACAAAACAATGC
Oligo-ARE-4W	F:TCGAGAAGAATTCTGTGAGAAAAA**ATTTA**ATAAAGCAACAAAAATTGC
	R:GGCCGCAATTTTTGTTGCTTTAT**TAAAT**TTTTTCTCACAGAATTCTTC
Oligo-ARE-4M	F:TCGAGAAGAATTCTGTGAGAAAAA**AGCA**ATAAAGCAACAAAAATTGC
	R:GGCCGCAATTTTTGTTGCTTTAT**TGCT**TTTTTCTCACAGAATTCTTC
Oligo-ARE-2W-1	F:TCGAGTTTCCTTAAAATTTTT**ATTTATTTA**CAACTCAAAAAACATTGGC
	R:GGCCGCCAATGTTTTTTGAGTTG**TAAATAAAT**AAAAATTTTAAGGAAAC
Oligo-ARE-2W-2	F:TCGAGTTTCCTTAAAATTTTT**ATTTATTTA**CAACTCAAAAAACATTGTTTTGTGTAAGC
	R:GGCCGCTTACACAAAACAATGTTTTTTGAGTTG**TAAATAAAT**AAAAATTTTAAGGAAAC
Oligo-ARE-2W-3	F:TCGAGCCTAATTTGGTTTCCTTAAAATTTTT**ATTTATTTA**CAACTCAAAAAACATTGGC
	R:GGCCGCCAATGTTTTTTGAGTTG**TAAATAAAT**AAAAATTTTAAGGAAACCAAATTAGGC

Underlined sequences are restriction enzyme sites.

(Thermo Pierce Biotechnology Scientific) as described previously [23]. Briefly, 20 fmol of biotinylated RNA was combined with 5 μg of cytoplasmic protein of cellular extract in a binding buffer. For the supershift EMSA, anti-V5 (20-783-70389, GenWay), anti-BRF1 (ab42473, Abcam), anti-Dcp2 (ab28658, Abcam) and anti-Xrn1 (ab70259, Abcam) antibodies were added to the reaction mixtures. After the addition of antibodies, reaction mixtures were incubated overnight on ice. The reaction mixtures were resolved on 5% nondenaturing polyacrylamide gels in 0.5 X Tris borate/EDTA buffer. Gels were transferred to nylon membrane (Hybond™-N^+) in 0.5 X Tris borate/EDTA at 70 V and 4 °C for 40 min. The RNAs were cross-linked to the membrane and detected using streptavidin-horseradish peroxidase binding and chemiluminescence.

2.5. SDS–PAGE analysis and immunoblotting

Proteins were resolved by SDS–PAGE, transferred to Hybond-P membranes (GE Healthcare Bio-Sciences Corp. Piscataway, NJ), and probed with the appropriate dilution of anti-V5 antibody. Immunoreactivity was detected using the ECL detection system (GE Healthcare Bio-Sciences Corp.). Films were exposed at multiple time points to ensure that images were not saturated.

2.6. Quantitative real-time PCR

For RNA kinetic analysis, we used actinomycin D and assessed *cIAP2* mRNA expression by quantitative real-time PCR. Quantitative real-time PCR was performed using the ABI Prism 7900 HT by monitoring in real-time the increase in fluorescence of the SYBR Green dye (QIAGEN, Hilden, Germany). Specificity of each primer pair was confirmed by melting curve analysis and agarose gel electrophoresis. PCR primer pairs were as follows: qcIAP2: AAGAGCTACTCGCCATACGCCTTT, AGCTTTGGCCATTTCTTGCTCCAG; qGAPDH: ATCTTCAAGCCATCCTGTGTGC, TGCGCTTGTCACATTTTTCTTG.

2.7. Statistics

For statistical comparisons, p values were determined using Student's t-test.

3. Results

3.1. TTP overexpression reduces the expression level of cIAP2 through destabilization of cIAP2 mRNA

In human, the TPA-inducible sequence 11 (TIS11) family consists of TTP, BRF1 and BRF2 and appears to interact with ARE-containing mRNAs and promote their degradation [26]. Previously, we found that BRF1 down regulates the expression of cIAP2 [23]. To determine whether TTP also reduces cIAP2 expression, we transiently transfected HeLa cells with pcDNA6/V5-TTP. As a negative control, HeLa cells were transfected with an empty pcDNA6/V5 vector. Expression levels of TTP and cIAP2 in HeLa cells transfected with pcDNA6/V5-TTP (HeLa/TTP) or pcDNA6/V5 (HeLa/pcDNA6) were analyzed by real-time PCR and RT-PCR. Our results show that overexpression of TTP significantly reduced the expression of cIAP2 (Fig. 1A and Fig. S1).

We next determined whether TTP overexpression decreases the stability of *cIAP2* mRNA. The half-life of *cIAP2* mRNA was measured in actinomycin D-treated HeLa/TTP and HeLa/pcDNA6 cells by quantitative real-time PCR. In HeLa cells, *cIAP2* mRNA was stable until 3 h after actinomycin treatment. However, in HeLa/TTP cells, the half-life was reduced to less than 3 h (Fig. 1B). Overall, these data indicate that the elevated TTP expression contributes to a decrease in cIAP2 levels through destabilization of *cIAP2* mRNA.

3.2. Overexpression of TTP decreases the expression level of luciferase mRNA containing the cIAP2 3′UTR

Analysis of the 640 bp human *cIAP2* 3′UTR revealed the presence of four ARE motifs (Fig. 2A). To determine whether the 3′UTR of *cIAP2* mRNA is required for TTP-mediated destabilization of *cIAP2* mRNA, we transfected 293 EBNA cells with a luciferase reporter gene linked to the *cIAP2* 3′UTR fragment containing all four AREs, Frag-ARE-1-4, in the plasmid psiCHECK. When 293 EBNA cells were transfected with pcDNA6/V5-TTP to overexpress TTP, the luciferase activity was dramatically inhibited in a dose dependent manner compared to that of 293 EBNA cells transfected with the empty vector pcDNA6/V5 (Fig. S2 A). The expression level of exogenous TTP in 293 EBNA cells is illustrated in Fig. S2 B. The results suggest that the 3′UTR of *cIAP2* mRNA is responsible for destabilization of *cIAP2* mRNA by TTP.

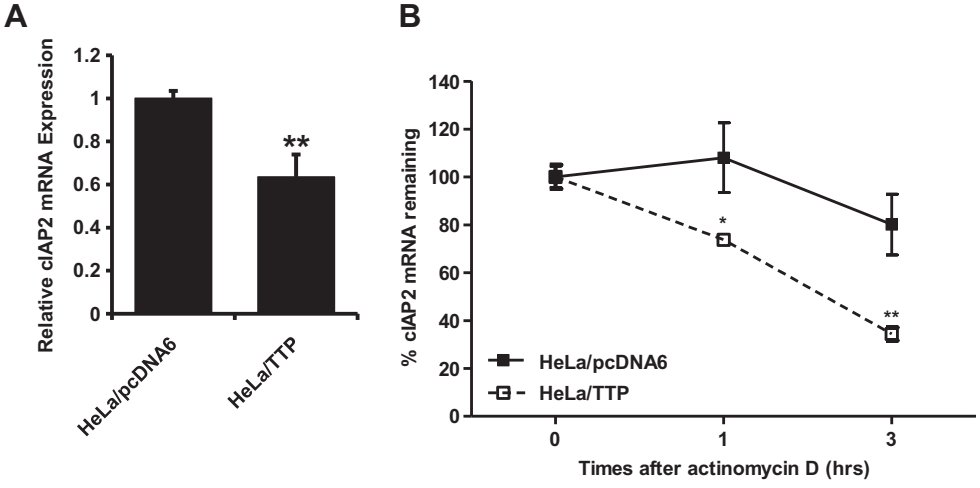

Fig. 1. Overexpression of TTP inhibits cIAP2 expression. (A) HeLa cells were transiently transfected with pcDNA6/V5-TTP or empty vector pcDNA6/V5. Expression levels of TTP and cIAP2 were determined by quantitative real-time PCR. GAPDH was detected as a loading control ($^{**}P < 0.005$). (B) Expression of *cIAP2* mRNA in HeLa cells was determined by quantitative real-time PCR at indicated times after the addition of 5 μg/ml actinomycin D. ($^{*}P < 0.05$, $^{**}P < 0.005$). Results shown on the graph represent means ± SD of three independent experiments.

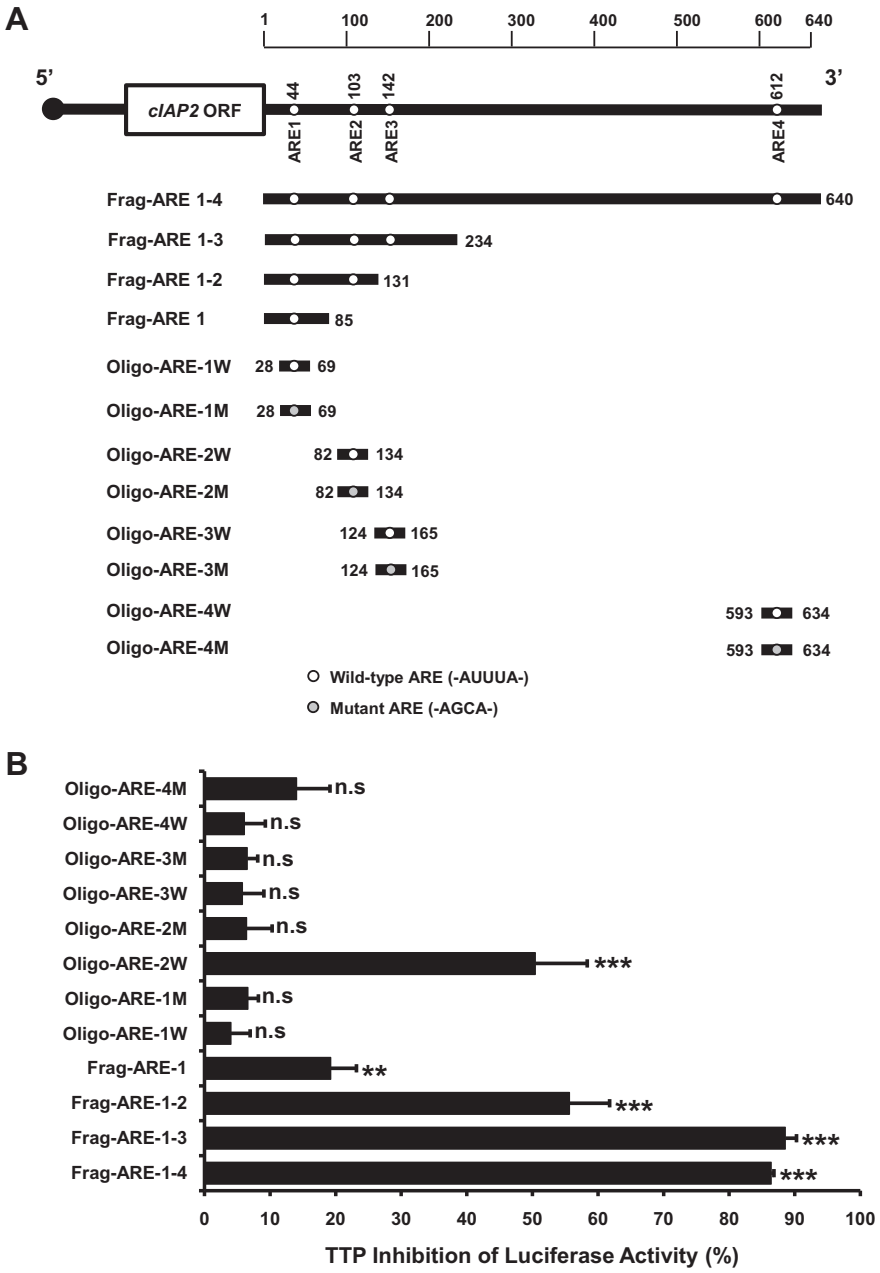

Fig. 2. The second ARE (ARE2) within *cIAP2* mRNA 3′UTR is responsible for TTP's inhibitory activity. (A) Schematic representation of the luciferase reporter constructs used in this study. Fragments (Frag) and oligonucleotides (Oligo) derived from the *cIAP2* mRNA 3′UTR were cloned downstream of the luciferase reporter gene in the psiCHECK2 luciferase expression vector. White circles, the wild-type (W) pentameric motif AUUUA; gray circles, the mutated (M) motif AGCA. (B) 293 EBNA cells were co-transfected with pcDNA6/V5-TTP and a psiCHECK2 luciferase reporter construct containing various fragments or oligonucleotides derived from the *cIAP2* mRNA 3′UTR as described in (A). After normalizing luciferase activity, TTP-induced inhibition of luciferase activity observed with each construct was compared to that obtained with the empty vector pcDNA6/V5. Results shown represent means ± SD of three independent experiments ($^{**}P < 0.005$; $^{***}P < 0.0005$).

3.3. The second ARE within cIAP2 3′UTR is essential for TTP inhibitory effect

To analyze which ARE motifs within the *cIAP2* mRNA 3′UTR are required for the TTP inhibitory effects on *cIAP2* mRNA stability, we first made use of luciferase genes linked to various deletion mutants of the *cIAP2* 3′UTR (Fig. 2A). While TTP decreased the luciferase activity of the luciferase reporter gene cloned upstream of Frag-ARE-1-4 (containing all four AREs), Frag-ARE-1-3 (containing ARE1, ARE2, ARE3) and Frag-ARE-1-2 (containing ARE1 and ARE2) by 82.7%, 87.5% and 55%, respectively, Frag-ARE-1 (containing ARE1) abrogated the inhibitory effect of TTP on the reporter gene activity (18% inhibition) (Fig. 2B). These results suggest that ARE2, ARE3, and/or ARE4 within the *cIAP2* 3′UTR are responsible for the inhibitory effect of TTP. To more accurately determine which ARE sequence is responsible for TTP activity, we prepared oligonucleotides: Oligo-ARE-1W (containing wild-type ARE1), Oligo-ARE-2W (containing wild-type ARE2), Oligo-ARE-3W (containing wild-type ARE3), and Oligo-ARE-4W (containing wild-type ARE4). We also prepared mutant oligonucleotides (Oligo-ARE-1M–Oligo-ARE-4M) in which AUUUA sequences were substituted with AGCA. While Oligo-ARE1W, Oligo-ARE-3W, and Oligo-ARE-4W did not respond to TTP, Oligo-ARE-2W responded to TTP (52% inhibition) (Fig. 2B). None of the mutant oligonucleotides responded to

TTP. These data suggest that the second ARE within the *cIAP* 3′UTR is involved in the TTP inhibitory activity.

3.4. TTP interacts with the second cIAP2 mRNA ARE

To determine the interaction of TTP with the second ARE of *cIAP2* 3′UTR, RNA EMSA was conducted using a biotinylated RNA probe containing the wild-type or mutant ARE2 of *cIAP2* mRNA. The RNA probe (Oligo-ARE 2W-1) used for RNA EMSA was 10 nucleotides shorter than the Oligo-ARE-2W oligonucletotide used for the luciferase assay (5 nucleotides deletion from both 5′ and 3′ ends of the Oligo-ARE-2W). A mutant oligonucleotide (Oligo-ARE-2M-1) was generated by substituting the AUUUA sequences with AGCA. Cytoplasmic extracts were prepared from 293 EBNA cells transfected with pcDNA6/V5-TTP to overexpress TTP and were incubated with biotinylated RNA probes, Oligo-ARE-2W-1 or Oligo-ARE-2M-1. When RNA EMSA was conducted using the wild-type Oligo-ARE-2W-1 probe, RNA–protein complexes were observed. However, the mutant Oligo-ARE-2M-1 probe prevented the formation of the complexes. Also, the RNA–protein complexes were super-shifted by pre-incubation of the reaction mixture with an anti-V5 antibody (Fig. S2 C). The results demonstrate that TTP can directly interact with the Oligo-ARE-2W-1 probe containing the second ARE of *cIAP2* 3′UTR.

3.5. TTP fails to decrease the expression level of luciferase mRNA containing the short Oligo-ARE-2W-1

In the RNA EMSA assay, we used a RNA probe (Oligo-ARE-2W-1), which is 10 bp shorter than the Oligo-ARE-2W probe used for TTP inhibitory activity analysis. Thus, we wanted to know whether this shorter oligonucleotide (Oligo-ARE-2W-1) can also respond to TTP. We subcloned this oligonucleotide, Oligo-ARE-2W-1, into the psiCHECK2 vector and compared its response to TTP with that of Oligo-ARE-2W. Interestingly, TTP failed to decrease the luciferase activity of the reporter gene cloned upstream of Oligo-ARE-2W-1. To determine whether the size of the oligonucleotide is important for the response to TTP, we prepared two more oligonucleotides, Oligo-ARE-2W-2 and Oligo-ARE-2W-3, which contain additional 10 nucleotides at the 3′ and 5′ ends of Oligo-ARE-2W-1, respectively (Fig. 3A). As shown in Fig. S3, both of them did not respond to TTP, suggesting that five additional nucleotide sequences from both ends of Oligo-ARE-2W-1 are required for TTP inhibitory activity.

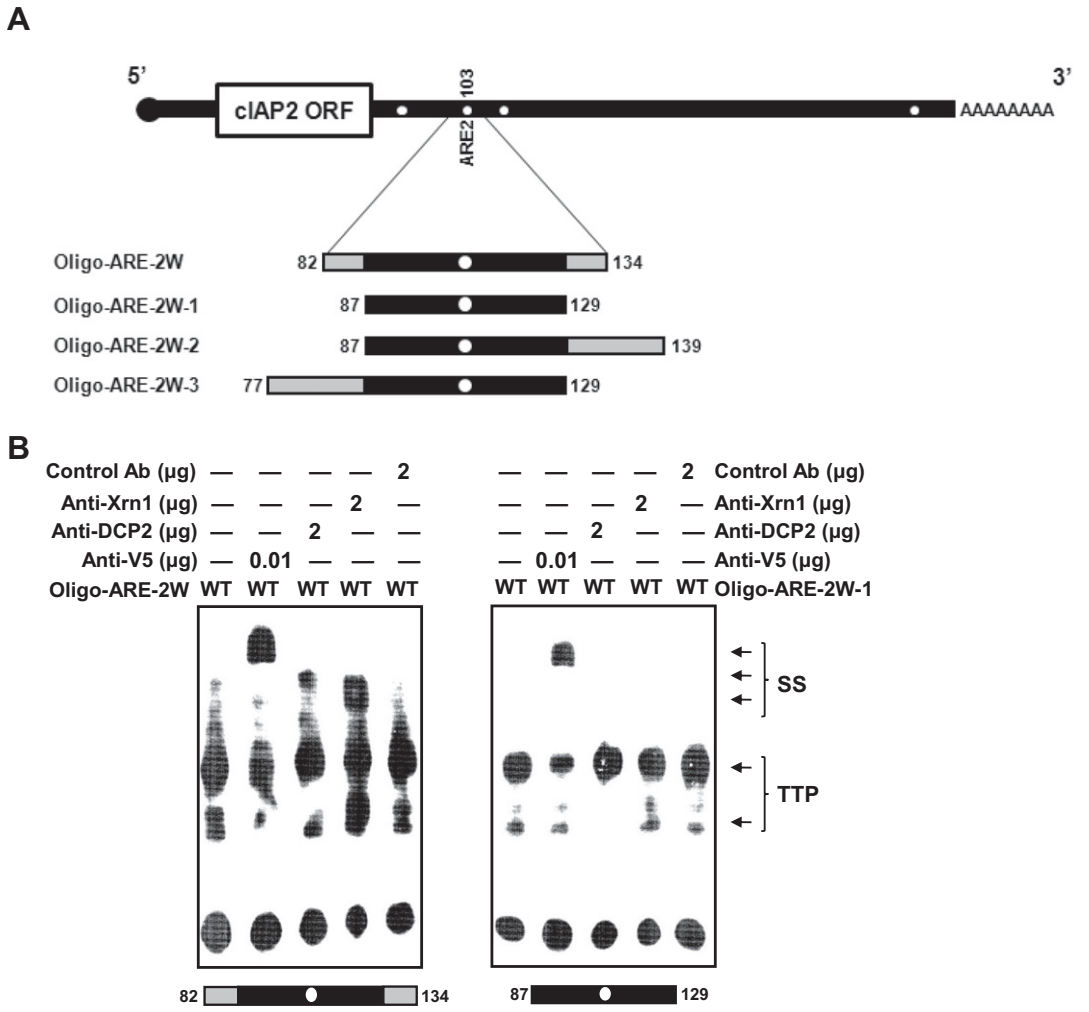

Fig. 3. TTP binding is not enough to recruit Dcp2 and Xrn1 and to induce decay of mRNA containing *cIAP2* 3′UTR. (A) Schematic representation of Oligo-ARE-2W, Oligo-ARE-2W-1, Oligo-ARE-2W-2, and Oligo-ARE-2W-3 used in this study. (B) Association of Dcp2 and Xrn1 with TTP-RNA complexes. An EMSA was performed as described in Section 2. Biotinylated Oligo-ARE-2W (left panel) or Oligo-ARE-2W-1 (right panel) was used as a probe. The following antibodies were added to the reaction mixtures: no antibody, control antibody, anti-V5 antibody, anti-Dcp2 antibody, and anti-Xrn1 antibody. The position of the TTP-containing bands (TTP) and super-shifted bands (SS) are indicated.

3.6. Five additional nucleotide sequences from both ends of Oligo-ARE-2W-1 are required for recruitment of RNA decay enzymes

To determine whether the two oligonucleotides, Oligo-ARE-2W and Oligo-ARE-2W-1 differ in their interaction with TTP, we used RNA EMSA. Even though there was a slight difference in the density of RNA–protein complexes, TTP formed complexes with both oligonucleotide probes (Fig. 3B). TTP-mediated mRNA decay requires the recruitment of protein components of P-body to the RNA–protein complexes [22,24]. Thus, our next goal was to determine the presence of P-body protein components, Dcp2 and Xrn1, in the RNA–protein complexes by preincubating the RNA–protein complexes with anti-Dcp2 and anti-Xrn1 antibodies. As illustrated in Fig. 3B, both anti-Dcp2 and anti-Xrn1 antibodies produced high molecular weight antibody complexes when the Oligo-ARE-2W probe was used (Fig. 3B, left panel). However, high molecular weight antibody complexes were not formed with Oligo-ARE-2W-1 (Fig. 3B, right panel). These data indicate that the additional 10 nucleotide sequences in the Oligo-ARE-2W probe play an important role in recruiting Dcp2 and Xrn1 to the RNA-TTP complexes and in TTP-mediated destabilization of *cIAP2* mRNA.

4. Discussion

cIAP2 has been shown to influence cell growth through the control of apoptosis and NF-kB signaling pathways and, thus, the expression of cIAP2 is tightly regulated. In this study we provide evidence that TTP suppresses the expression of cIAP2 through destabilization of *cIAP2* mRNA. Overexpression of TTP reduces the stability of *cIAP2* mRNA and the expression level of endogenous cIAP2. Insertion of the *cIAP2* 3′UTR sequences into a luciferase reporter construct conferred TTP-mediated mRNA destabilization on the luciferase reporter, suggesting that *cIAP2* 3′UTR is responsible for TTP-mediated destabilization of *cIAP2* mRNA. The 3′UTR of *cIAP2* mRNA contains four AREs and we have shown that, among them, the second ARE is essential for TTP binding and TTP-mediated destabilization. Collectively, these results suggest that TTP regulates *cIAP2* gene expression through direct binding to the 2nd ARE within the *cIAP2* 3′UTR and inducing its decay.

Previously, we reported that BRF1 suppresses the expression of cIAP2 [23]. Combined with our previous report [23], our results in the present study suggest that both TTP and BRF1 target *cIAP2* mRNA for rapid decay. Like TTP, BRF1 destabilizes *cIAP2* mRNA through binding to the second ARE of *cIAP2* mRNA (Figs. 1 and 2 and Figs. S1, S2 and S4). It is not known why cells use two different proteins, TTP and BRF1, for the post-transcriptional control of *cIAP2*. One possible reason is that cIAP2 expression is critical for the control of cell growth and, thus, cells have multiple regulators of cIAP2 expression for redundancy. Several reports support this possibility: both TTP and BRF1 appear to activate the decay of ARE-containing mRNAs by recruiting the same mRNA decay enzymes [21,22]; their mRNA decay activities are impaired by phosphorylation followed by binding to 14-3-3 [27–30]. However, the protein kinases responsible for phosphorylation of TTP and BRF1 are different: TTP is phosphorylated by MK-2 [29,30] and BRF1 by protein kinase B [27,28]. This suggests that TTP and BRF1 are not only for redundancy but may be used to respond to different signals that require down-regulation of cIAP2.

TTP accelerates the decay of ARE-containing transcripts through direct binding to the ARE of target transcripts. TTP exhibits no enzymatic activity itself but mediates decay of the target transcripts by recruiting various components of the basic RNA decay machinery such as Dcp2 and Xrn1. [31] reported that short (22–32 nucleotide) AU-rich high affinity TTP binding sites are sufficient to allow TTP-dependent mRNA decay and suggested a model whereby TTP binding is all that is necessary in order for an ARE to be functional. However, our observations suggest that TTP binding is not enough for the TTP-mediated decay of the *cIAP2* ARE-containing transcript. First, the 42 bp sequence containing the 2nd ARE of *cIAP2* mRNA are sufficient for TTP binding but not for recruiting Dcp2 and Xrn1 nor TTP-mediated decay (Figs. S2 C and S3, and Fig. 3B). Second, the 52 bp sequence containing additional 10 bp of *cIAP2* 3′UTR are able to form TTP-RNA complexes containing Dcp2 and Xrn1 and induce TTP-mediated decay of transcripts (Fig. S3, and Fig. 3B). Our results suggest that besides the nucleotide sequences for the TTP binding, additional nucleotide sequences are necessary for recruitment of Dcp2 and Xrn1 and for the TTP-mediated decay of *cIAP2* mRNA. Our results are consistent with previous reports demonstrating that while Dcp2 does not efficiently decap short mRNA substrates, long ones are efficiently hydrolyzed [32,33]. Based on our results, there seems to be a sequence specific interaction between *cIAP2* 3′UTR and Dcp2 and/or Xrn1 because, while addition of 10 bps to the 5′ or 3′end of the 42 nucleotides did not induce TTP-mediated mRNA decay, addition of 5 nucleotides to both the 5′ and 3′end of the 42 nucleotides did induce TTP-mediated mRNA decay.

In this report, we have shown that TTP negatively regulates cIAP2 expression through the destabilization of *cIAP2* mRNA. TTP-mediated mRNA decay has been reported to require the recruitment of Dcp2 and Xrn1 [22,24]. Interestingly, TTP binding is not enough to recruit Dcp2 and Xrn1 and initiate decay of mRNA containing *cIAP2* 3′UTR. Instead, several additional nucleotide sequences are required to recruit Dcp2 and Xrn1 and induce the decay of *cIAP2* mRNA. Further studies will need to address how the additional sequences within the *cIAP2* 3′UTR recruit Dcp2 and Xrn1, and the precise role of TTP in this process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.07.136.

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