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Ubiquitination of mRNA cycling sequence binding protein from *Leishmania donovani* (LdCSBP) modulates the RNA endonuclease activity of its Smr domain

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ABSTRACT

In trypanosomatid parasites, an octanucleotide sequence (C/A)AUAGAA(G/A) in the UTRs primarily determines the stability of S-phase specific mRNAs. A multi-domain protein LdCSBP from *Leishmania donovani* interacts with the UTR of an S-phase RNA containing the octanucleotide sequence through its unique CCCH-type Zn-finger motifs. Interestingly, the RNA binding protein contains a previously characterized DNA endonuclease domain – Smr. It has been demonstrated here that the LdCSBP Smr domain independently possesses both DNA and RNA endonuclease activities, but the full-length LdCSBP exhibits only riboendonuclease activity. Moreover, LdCSBP protein has been shown to be ubiquitinated, resulting in the down-regulation of its riboendonuclease activity. In conclusion, the results described here suggest a novel regulatory mechanism of mRNA degradation through ubiquitination in eukaryotes.

1. Introduction

In all life forms, RNA degradation is an essential cellular activity that maintains the appropriate level of mature species of various classes of RNAs and removes the aberrant ones. In eukaryotes, stabilities of specific mRNAs are defined by the presence or absence of particular *cis*-elements within their UTRs. The most studied instability determinants are the AU-rich elements (ARE) and Tristetraprolin (TTP) is an ARE binding protein required for rapid degradation of mRNAs encoding cytokines such as TNFα, GM-CSF and IL-2 [1]. The ARE-binding proteins can recruit mRNA-decay factors resulting in the enhancement of degradation.

Interestingly, in early branching unicellular flagellated trypanosomatid parasites, many species of which are highly pathogenic, there is nearly complete lack of transcriptional regulation of gene expression and mRNA turnover is the main mode of regulation of differential gene expression. Several unique *cis*-elements responsible for stage and cell cycle specific gene expression have been identified in the parasites. One such element is the consensus octameric sequence (C/A)AUAGAA(G/A) present in 5′- or 3′-UTR of the transcripts that are periodically stabilized during S-phase of cell cycle [2,3]. Two protein complexes – CSBP and CSBP-II, have been identified in *Crithidia fasciculata*, which bind periodically to the octameric sequences of the transcripts with high specificity [4–6]. We have previously reported the characterization of the mRNA cycling sequence binding protein from *Leishmania donovani* (LdCSBP), which specifically binds to the octamer containing RNA [7]. The LdCSBP and its orthologs in related trypanosomatids contain two ubiquitin related domains UBA and CUE suggesting a ubiquitination-mediated regulatory mechanism for its cellular function. Strikingly, unlike the *C. fasciculata* counterparts, LdCSBP contains a small MutS-related (Smr) DNA endonuclease domain [7], which has been initially found in bacterial MutS2 protein [8,9] and later identified in the mammalian Bcl3 binding protein (B3BP) and plant PAM-2 that are not members of MutS family [10,11]. It is intriguing to note that LdCSBP, which is a specific RNA binding protein, contains a DNA endonuclease domain [7] raising the possibility of involvement of the Smr domain in RNA degradation. Such an interesting possibility is addressed in the present study where we demonstrate for the first time that the Smr domain of LdCSBP possesses RNA endonuclease activity. Furthermore, we also show that the RNA endonuclease activity of the protein is inhibited by ubiquitination, implicating a novel regulatory mechanism of mRNA turnover.

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2. Materials and methods

2.1. Protein expression in Escherichia coli

For expression of the individual domains of LdCSBP with N-terminal GST-tag (Supplementary Fig. S1 and Fig. 1A) in bacteria, the corresponding DNA fragments were cloned into pGEX-5X-3 vector and the chimeras were expressed in *E. coli* strain BL21 (DE3) and purified over glutathione Sepharose beads (GE Healthcare).

2.2. RNA electrophoretic mobility shift assay (REMSA)

REMSA was carried as described before [7] using the wild type and mutated octamer motif containing LdCyc1 5′ RNAs (Supplementary Fig. S2).

2.3. DNA endonuclease assay

DNA endonuclease assay was carried out at 37 °C in a total volume of 10 μl with 0.4 μM of enzyme and 350 ng pRSET-C plasmid as substrate and the products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

2.4. RNA endonuclease assay

5′-end labeling of 20-mer RNA substrate was performed with T4-PNK (NEB) and [γ-^{32}P]ATP. Circular RNA substrate was prepared from the linear 5′ labeled 20-mer RNA by using T4 RNA Ligase (Epicentre). 3′-end labeling was carried out as described before [12]. The endonuclease assay was carried out with the radiolabeled RNAs at 30 °C and the products were analyzed on a 20% polyacrylamide gel containing 8 M urea.

2.5. Polyubiquitin binding assay

Bacterially expressed GST and GST-tagged domains of LdCSBP (10 μg each) were incubated individually for overnight with 5 μg K48 linked His–Ub_{2-7} (Boston Biochem^{TM}) bound to Ni-NTA Agarose beads in a total volume of 200 μl at 4 °C. The pulled-down complexes were analyzed by immunoblotting using monoclonal antibodies against GST (Santa Cruz Biotechnology) and ubiquitin (Imgenex).

2.6. Ubiquitination of LdCSBP

Ubiquitination was carried out using S-100 HeLa conjugation kit (Boston Biochem^{TM}), replacing the HeLa S-100 extract with *L. donovani* S-100 extract. A control reaction was always carried out exactly in similar condition, but without the addition of the energy regeneration system and ubiquitin. It was routinely observed that the integrities of the target proteins were intact in both control and experimental reactions.

3. Results and discussion

3.1. LdCSBP interacts specifically with UTR of an S-phase mRNA through its unique CCCH Zn-finger motifs

In our earlier report it was shown that LdCSBP specifically interacted with an RNA fragment containing six copies of the CAU-

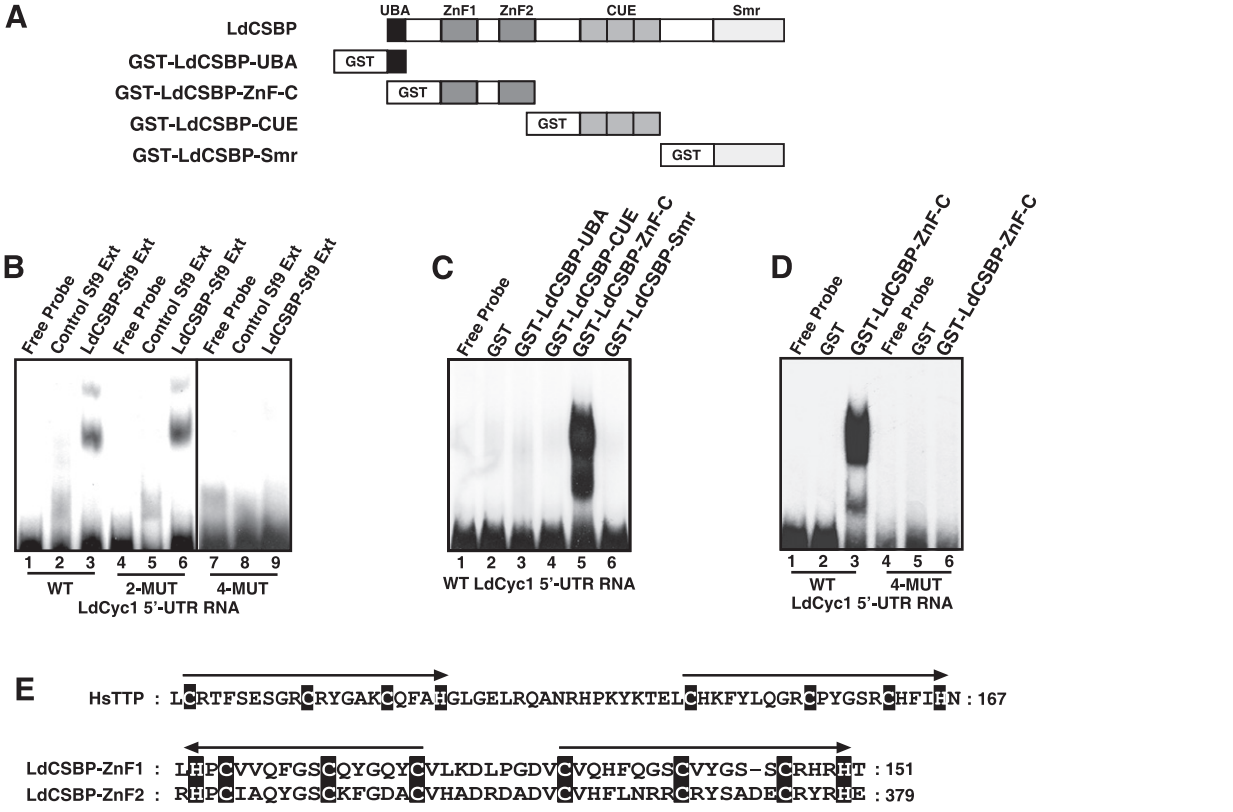

Fig. 1. Specific binding of LdCSBP with S-phase cyclin LdCyc1 5′-UTR RNA through its CCCH Zn-Finger motifs. (A) Schematic representation GST fusion domains of LdCSBP used in the subsequent studies. (B) REMSA with ^{32}P-uridine labeled LdCyc1 5′-UTR RNA containing octamer with no mutation (WT, CAUAGAAG) (lanes 1–3), two (2-Mut, CC*G*AGAAG) (lanes 4–6) or four mutations (4-Mut, CC*GTC*AAG) (lanes 7–9) and control Sf9 insect cell extract (15 μg total protein; lanes 2, 5 and 8) or extract of Sf9 cells expressing LdCSBP (15 μg total protein; lanes 3, 6 and 9). (C) REMSA with LdCyc1 5′-UTR RNA containing wild type octamer and purified GST as control (lane 2) or GST fusion domains (750 nM each) of LdCSBP (lanes 3–6). (D) REMSA with LdCyc1 5′-UTR RNA containing wild type (lanes 1–3) or mutated octamer (lanes 4–6) and GST (750 nM; lanes 2 and 5) or GST-ZnF-C (750 nM; lanes 3 and 6). (E) CCCH Zn finger motifs (conserved Cys and His residues are highlighted in black) of human tristetraprolin (HsTTP) and LdCSBP.