The trichomonad cysteine proteinase TVCP4 transcript contains an iron-responsive element


Department of Biotechnology and Bioengineering, Centro de Investigación y Estudios Avanzados del IPN (CINVESTAV-IPN), Av. IPN # 2508, Col. San Pedro Zacatenco, CP 07360, Mexico City, Mexico

Department of Experimental Pathology, Centro de Investigación y Estudios Avanzados del IPN (CINVESTAV-IPN), Av. IPN # 2508, Col. San Pedro Zacatenco, CP 07360, Mexico City, Mexico

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Abstract The differential expression of the *Trichomonas vaginalis* cysteine proteinase TVCP4 by iron at the protein synthesis level and the prediction of an iron-responsive element (IRE)-like stem-loop structure at the 5′-region of the *T. vaginalis* cysteine proteinase gene (tvcp4) mRNA suggest a post-transcriptional mechanism of iron regulation in trichomonads mediated by an IRE/IRP-like system. Gel-shifting, UV cross-linking and competition experiments demonstrated that this IRE-like structure specifically bound to human iron regulatory protein-1. IRE-like cytoplasmic proteins that bound human ferritin IRE sequence transcripts at low-iron conditions were also found in trichomonads. Thus, a post-transcriptional regulatory mechanism by iron for tvcp4 mediated by IRE/IRP-like interactions was found. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

Iron is an essential nutrient for growth, metabolism, and virulence of parasitic protozoa including *Trichomonas vaginalis* [1]. The environment of the human vagina, especially its nutrient and iron concentration, is constantly changing throughout the menstrual cycle. *T. vaginalis* may respond to varying iron concentrations by differential gene expression regulation through a poorly understood mechanism in order to survive, grow and colonize the vaginal hostile environment.

Iron regulates the enhanced expression of some hydrogenosomal proteins [2], phosphohydrolases [3], and virulence genes encoding the trichomonad adhesins AP65 (decarboxylating malic enzyme), AP51 (β-Succinyl Coenzyme A synthetase, SCS), AP33 (α-SCS), and AP120 (pyruvate:ferredoxin oxidoreductase) [4–7]. High-iron concentrations also induce *T. vaginalis* resistance to complement lysis through expression of proteinases that degrade the C3b component [8]. In contrast, transcription of the *T. vaginalis* cysteine proteinase 12 gene (tvcp12), and the flp-1 and flp-2 genes of fibronectin-like proteins are downregulated by iron [9,10], as well as the expression of the *tvcp65* gene, which negatively affects trichomonadal cytotoxicity (our unpublished data).

An iron-responsive promoter and other regulatory elements in the 5′-upstream region of the *ap65-1* gene were identified as a mechanism for positive transcriptional regulation of trichomonad genes by iron [11–13]. However, up to now, this iron-responsive promoter has not been found in other gene sequences coding iron-regulated proteins. Thus, it is likely that other iron regulatory mechanisms may exist in this parasite, possibly at the post-transcriptional level, like the one that mediates the coordinated regulation of key proteins of iron metabolism, such as ferritin, transferrin receptor and erythroid 5-aminolevulinic acid synthase [14,15]. This post-transcriptional mechanism involves an interaction between trans-acting cytoplasmic iron regulatory proteins (IRP-1 and IRP-2), and cis-acting iron-responsive elements (IREs) present within the 5′-or 3′-untranslated regions (UTR’s) of some mRNAs, the IRE/IRP system. Binding of IRE by IRP could inhibit mRNA translation or degradation, depending on the 5′- or 3′-UTR’s location [14–17].

*T. vaginalis* contains many cysteine proteinases (CPs) [18]. Some CPs participate in virulence, such as CP30 and CP62, involved in trichomonadal cytoadherence [19–21]; whereas, CP65 and CP39 participate in trichomonal cytotoxicity [22,23]. Furthermore, expression and proteolytic activity of several trichomonadal CPs are modulated by iron, especially at the ~25- to 35-kDa region [10].

In the present study, we found that expression of the cysteine proteinase TVCP4 is upregulated by iron at the post-transcriptional level probably through an IRE/IRP-like system, as in other organisms. To test this hypothesis, binding of an IRE-like hairpin secondary structure of the *tvcp4* mRNA to human IRP proteins, as well as presence of trichomonadal IRE-like cytoplasmic proteins, which bind to human ferritin IRE sequence (IRE-fer) transcript, is shown. Information derived from this study will be useful for further elucidating alternative
mechanisms of gene expression regulation by iron in *T. vaginalis*.

2. Materials and methods

2.1. Parasites and culture conditions

Trichomonads of *T. vaginalis* isolate CNCD 147 were grown in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% (v/v) heat-inactivated horse serum [24] containing ∼20 μM iron [2]. Parasites in the logarithmic growth phase were grown either in iron-rich or in iron-depleted medium by the addition of 250 μM ammonium ferrous sulfate, or 150 μM 2'-dipyridyl, an iron-chelator, respectively (Sigma Co., St Louis, MO, USA) into the culture medium [8,9].

2.2. Analysis of *tvcp4* gene sequence

The genomic clone 2.9.1.1 [10] with a 3057-bp insert was sequenced (GenBank Accession No. AY679763). This clone contains a 915-bp open reading frame (ORF) encoding a complete CP gene. Its DNA sequence was analyzed by BLAST, WORKBENCH and EXPASY search engines. Locations of DNA transcription-factor binding motifs and predicted RNA secondary structures were analyzed using the Transfac program (http://www.gene-regulation.com/pub/programs/allibaba2/index.html) and the mfold program (http://bioinfo.rpi.edu/applications/mfold/), respectively [25].

2.3. RT-PCR assays

RT-PCR assays were performed using the Superscript RNase H Reverse Transcriptase kit (Stratagene), as recommended by the manufacturer. Total RNA prepared using TRIzol reagent from parasites grown in iron-rich or in iron-depleted medium by the addition of 250 μM ammonium ferrous sulfate, or 150 μM 2'-dipyridyl, an iron-chelator, respectively (Sigma Co., St Louis, MO, USA) was reverse-transcribed using an AMV reverse transcriptase and the oligo (d)T primer. Then, a fragment of 689-bp of the *tvcp4* cDNA was amplified by PCR using as primer CP4 5'-GCTAACCCTGCCCTACA-3' at position 139–156 nt, and as antisense primer CP4 5'-CCCGAGGTGACACGTAC-3' at position 800–819 nt. A 112-bp fragment of the *T. vaginalis* *β*-tubulin gene was amplified by PCR with BTUB9 primer: 5'-CATGATAACGAGGCTCTTACGAT-3' and BTUB2 primer: 5'-GCATGTGGTGCCGCAGACATAACC-3', and used as an internal control [10].

2.4. Western blot analysis

Total trichomonad proteins from 2 × 107 parasites grown in iron-rich and iron-depleted medium obtained as before [26], recombinant human iron regulatory protein (rhirP-1) [27–29], or HeLa cell cytoplasmic extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. Duplicated gels were transferred onto nitrocellulose membrane for Western analysis. Specific proteins were immunodetected with mouse polyclonal antibodies to a synthetic peptide (NAAKGTS-WIKS) selected from the most divergent region (residues 188–197) of the TVCP4 proteinase (anti-TVCP4 at 1:12000 dilution), to the recombinant human ferritin H-chain (anti-IRE-fer) region, linearized with BamHI [28], three templates and unincorporated nucleotides were removed by DNase digestion and UV-induced cross-linking assays. RNA–protein complexes (RPCs) were resolved by SDS–PAGE on a non-denaturing polyacrylamide gel and visualized by autoradiography. UV-induced cross-linking assays.

2.5. Purification of recombinant human iron regulatory protein-1 (hirP-1)

Recombinant hirP-1 protein subcloned into pGEX-hirP plasmid, containing full-length cDNA (kindly donated by Dr. Lucas Kühn), was expressed and purified [27–29]. Purified protein samples were quantified by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Purity of protein samples was assessed by SDS-PAGE on Coomassie brilliant blue-stained gels. Purified rhIRP-1 was used as a control for RNA-band shift, UV-induced cross-linking, and Western blot assays, as well as antigen for polyclonal antibody production in mice (anti-IRP antibody).

2.6. Cytoplasmic extracts from HeLa cells and trichomonad parasites

HeLa cell cytoplasmic extracts were prepared by a modified method [31], and used for gel-shifting and UV cross-linking assays. Briefly, HeLa cells were washed with cold PBS and centrifuged (2500 × g for 5 min at 4 °C). The cell pellet was suspended in lysis buffer A (10 mM HEPES–NaOH, pH 7.9, 15 mM MgCl2, 10 mM KC1), homogenized in a Dounce homogenizer (45 strokes), and centrifuged (10000 × g for 30 min at 4 °C). Supernatants were diluted to a final protein concentration of 10 mg/ml and kept at −70 °C.

Trichomonad cytoplasmic extracts were prepared from 1 × 108 trichomonads grown in iron-rich or iron-depleted medium by a modified method [31]. Parasites were lysed by vortexing in 250 μl interaction buffer (10 mM HEPES, pH 7.6, 3 mM MgCl2, 40 mM KC1, 5% glycerol and 0.3% NP-40) containing 7.5 mM TLCK and 1.6 mM leupeptin, incubated for 20 min at 4 °C, and centrifuged at 13000 × g for 5 min at 4 °C. Then, the supernatant was recovered, protein concentration was determined by Bradford (Bio-Rad), and aliquots were kept at −70 °C.

2.7. In vitro transcription of IRE-like sequences

The DNA used for in vitro transcription included: pSPT-fer plasmid (a generous gift of Dr. Lucas Kühn) containing human ferritin H-chain IRE (IRE-fer) region, linearized with BamHI [28], three IRE-like hairpin sequences, ampiclon 94 from bp 0 to 28 (including the *tvcp4* IRE-like hairpin sequence), ampiclon 94 from bp –3 to –10 (containing the *tvcp4* IRE-like hairpin sequence), and ampiclon 97 from bp 12 to 107 (a deletion mutant that disrupts the *tvcp4* IRE-like hairpin sequence). Ampiclons were produced by PCR using primers: sense (31): 5'-TAAATGCACTATAGAACTGTCGATG-3' and antisense (31): 5'-CTTCTGCTAGCTTGATGGAGAACGAGACATGTTG-3'; sense (94) 5'-TAAATGCACTATAGAACTGTCGATG-3' and antisense (94) 5'-TCGCCGTGGAACATTTGC-3'; sense (97) 5'-TAAATGCACTATAGAACTGTCGATG-3' and antisense (97) 5'-GGAGGAGCAATATGCCCAGAAGACGACGAAAC-3', respectively. PCR sense primers contain a bacteriophage T7 promoter sequence (underline nt) and an additional sequence GG for enhancing transcription. Purified PCR products were used as templates for RNA synthesis using an in vitro transcription kit (Promega Corp. Madison, WI, USA). DNA templates and unincorporated nucleotides were removed by DNAse RQ1 (Promega) treatment in the presence of RNase inhibitors (Promega) and by gel filtration, respectively. For the synthesis of radiolabeled RNA transcripts, 20 μl mixtures contained 10 mM HEPES–KOH, pH 7.4, 3 mM MgCl2, 5% (v/v) glycerol and 0.3% NP-40 containing 7.5 mM DTT and 1 mM RNasin, incubated for 20 min at 4 °C, and centrifuged at 13000 × g for 5 min at 4 °C. Then, the supernatant was recovered, protein concentration was determined by Bradford (Bio-Rad), and aliquots were kept at −70 °C.

2.8. RNA band-shift assay

RNA–protein complexes were first detected by band-shift assays. [32P]labeled RNAs 0.2 ng (2 × 106 cpm) were incubated for 10 min at 25 °C with recombinant hirP-1 (1 μg/20 μl volume) in the presence of 5 μg heparin, and IRE-IRP complexes were resolved on a 6% non-denaturing polyacrylamide gel and visualized by autoradiography [28].

2.9. UV cross-linking assays

Recombinant hirP-1 (0.5–1.0 μg), 30 μg cytoplasmic extract of HeLa cells, or 50 μg cytoplasmic extract of *T. vaginalis* were incubated with [32P]labeled RNA probes (5 × 106 cpm) for 20 min at 30 °C in 25 μl reaction mixture containing 10 mM HEPES–KOH, pH 7.4, 3 mM MgCl2, 5% (v/v) glycerol, 1 mM DTT, 100 mM KCl, 40 U RNAsin and 6 μg yeast tRNA (Invitrogen Corp., Carlsbad, CA, USA). After RNA-binding, the reaction mixture was placed on ice and irradiated with a UV-lamp (240 nm) for 30 min. Samples were then incubated with RNase A (10 μg) and RNase T1 (20 U) for 30 min at 25 °C. RNA–protein complexes were resolved by SDS-PAGE on a 10% polyacrylamide gel. The gels were stained with Coomassie brilliant blue and dried, and radioactive bands were visualized by autoradiography.
For competition assays, a 10- and 20-fold molar excess of unlabeled 31-nt tvcp4 IRE-like transcript or IRE-fer RNA were incubated with cytoplasmic extracts for 15 min at 4 °C before the addition of labeled RNA. rRNA was used as a heterologous competitor [32]. Densitometry analysis of cross-linked bands was performed with the Quantity One program (Bio-Rad).

2.10. Immunoprecipitation of IRP-like proteins in the RPCs after UV cross-linking assays with an anti-IRP antibody

RPCs were incubated with 10 μl of protein G-Sepharose 4B beads (pre-equilibrated in lysis buffer A, for 2 h at 4 °C, and centrifuged at 12000 g for 5 min) (Invitrogen) after UV cross-linking and RNase treatment. The supernatants were then incubated with 6.0 μl of mouse polyclonal anti-recombinant hIRP-1 antibody for 18 h at 4 °C. These immunocomplexes were immobilized on protein G-Sepharose beads (30 μl) saturated with 2% bovine serum albumin, for 3 h at 4 °C. Unbound materials were eliminated by washing six times with 10 mM Tris–HCl buffer (pH 8.0), containing 150 mM NaCl, 1% (v/v) NP-40, and 3 mM EDTA. Bound proteins were eluted with Laemmli buffer and analyzed by SDS–PAGE, followed by autoradiography. Parallel reactions were carried out with the pre-immune normal mouse serum as a negative control [32].

3. Results

3.1. The complete T. vaginalis tvcp4 gene encodes a cathepsin L precursor proteinase

Genomic clone 2.9.1.1 (with a 3057-bp insert) contains a 915-bp ORF with an ATG initiation and TAA stop codons, non-coding regions up- and downstream of it containing the Inr promoter [33] and putative polyadenylation regulatory sequences, respectively [34]. The deduced protein (305-aa) is 99% identical to the partial tvcp4 cDNA sequence reported before [35]. TVCP4 protein corresponds to a putative papain-like precursor proteinase with a predicted molecular mass of 33.8-kDa and a theoretical pI of ~7.5. The putative TVCP4 precursor contains a signal peptide (SP, residues 1–25), pre- and pro-regions (residues 26–86), and an ERF-NIN-like sequence (white box, residues 26–45). The putative mature TVCP4 protein begins at residue 87 (arrowhead) with a predicted mass of 23.6-kDa and a theoretical pI of ~5.22.

Fig. 1. The complete T. vaginalis tvcp4 gene and effect of iron on transcription and translation of TVCP4. (A) Principal motifs of the deduced amino acid sequence of the TVCP4 protein, residues forming the putative signal peptide (SP), pre-pro region (small grey box), ERFNIN sequence (ERF, white box), (▼) putative start site of the mature CP, C, H and N, and the amino acid residues (Cys, His and Asp) forming the catalytic triad of the TVCP4 cysteine proteinase. (B) RT-PCR products obtained with specific primers for the tvcp4 (tvcp4), or β-tubulin (β-tub) gene using cDNA from parasites grown in: lane 1, iron-depleted (L); or lane 2, iron-rich (H) medium. (C) Densitometric analysis of the RT-PCR products from (B) using the QUANTITY ONE software. The intensity obtained from the β-tub product was used as 100%. (D) Western blot analysis using nitrocellulose membranes containing TCA precipitated total protein extracts from: lane 1, parasites grown in iron-depleted (L); or lane 2, iron-rich (H) medium. (E) Densitometric analysis of protein bands detected by Western blot from (D) using the QUANTITY ONE software. The band intensity obtained with the anti-TVCP4 over protein extracts from parasites grown in iron-rich medium (H) was used as 100%. Experiments were performed three times with similar results.
3.2. High iron trichomonads have increased amounts of TVCP4

We performed RT-PCR assays using specific primers for tvcp4, and RNA from parasites grown in iron-rich and iron-depleted medium. The expected 689-bp product was obtained with RNA from both iron conditions, showing only minor changes (Fig. 1B, tvcp4). As an internal control, we obtained identical products of 112-bp for the β-tubulin gene by RT-PCR with RNA from both iron conditions handled identically (part B, β-tub), which was used as 100% intensity in the densitometry scans (part C).

We performed Western blot with extracts from high- and low-iron parasites used above, and probed with anti-TVCP4 peptide serum antibody. Fig. 1 (parts D and E) shows 3-fold increased amounts of TVCP4 in iron-rich compared to iron-depleted parasites. As a control, similar amounts of protein were detected with antiserum to α-tubulin in both iron conditions. These data show that iron is without effect on gene transcription and is increasing translation of TVCP4.

3.3. An IRE-like structure is found inside the coding region of the tvcp4 mRNA

To investigate the mechanism by which amounts of TVCP4 are increased in high-iron parasites, we first analyzed a 1306-bp region of the tvcp4 5'-UTR, obtained from the draft T. vaginalis genome sequence [36] with the Transfac program. Typical regulatory sequences of trichomonad genes at the 5'-UTR, such as the Inr promoter element TCATTTC [33] located at 12-nt upstream of the initiation codon ATG, were observed (Fig. 2A). However, none of the reported DNA motifs were related to iron regulation previously described for ap65-1 gene [11–13]. Thus, we searched for iron regulatory elements (IREs) in the tvcp4 mRNA that could be involved in a post-transcriptional regulatory mechanism mediated by an IRE/IRP-like system [14–17]. Analysis by the mfold program [25] of 21-nt upstream and 107-nt downstream of the ATG codon of tvcp4 mRNA revealed a sequence with possible formation of stem-loop RNA secondary structures (Fig. 2A). The first 23-nt downstream of the ATG codon formed a stable (ΔG = -5.7 kcal/mol) IRE-like stem-loop structure with a 6-nt loop (part B3), a 5-nt upper stem, a bulge, and a 3-nt lower stem [14,28,29].

The predicted 23-nt tvcp4 IRE-like stem-loop RNA secondary structure was compared with the secondary structures of the human IRE-fer determined by NMR [37] (part B1), and with the IRE present in the mitochondrial 75-kDa subunit of mitochondrial complex I [38] (part B2). The 6-nt loop of the tvcp4 IRE-like hairpin has a 5'-GGCAAC-3' sequence, although it possesses a G1 and C5 nucleotide arrangement similar to the hairpin loop of IRE-fer mutants that bind to IRP proteins [28,29] and can be therefore classified as a G1/C5-type IRE element (part C). Even though the five-paired
nucleotide upper stem of the tvcp4 IRE-like hairpin is similar to the one present in the IRE-fer [14,15], the tvcp4 IRE-like structure has a bulge with an A at the 3'-strand as in the 75-kDa subunit of mitochondrial complex I, which is functional [28,29]. Moreover, the tvcp4 IRE-like lower stem is three-paired nt similar to the one found in the IRE of the 75-kDa subunit of mitochondrial complex I, while in the IRE-fer it is five-paired nt long. Interestingly, four out of the five-paired nt in the upper stem, and the three-paired nt of the lower stem of the tvcp4 IRE-like RNA predicted secondary structure are identical to the corresponding IRE-fer sequence (parts B1 and 3, bold letters).

3.4. The tvcp4 IRE-like hairpin structure interacts with the recombinant hIRP-1 and with a ~98-kDa protein band from HeLa cytoplasmic extracts

The ability of tvcp4 IRE-like hairpin to form RPCs with IRP-like proteins was then tested by gel-shifting and UV cross-linking assays (Fig. 3). We first analyzed the predicted secondary structure of different sized transcripts, 31- (–3 to 28), 94- (–10 to 84), and 97-nt (12–107) from the 5'-end of tvcp4 for the presence of IRE-like structures (Fig. 3A). Only the 97-nt RNA predicted secondary structure did not form an IRE-like structure. Thus, the 97-nt transcript was used as a deletion mutant with a disrupted IRE-like structure.

We next tested by gel-shifting assays using the recombinant hIRP-1 protein the ability of the different tvcp4 IRE-like transcripts to bind to IRP proteins. Upon incubation with the 32P-labeled 31- and 94-nt tvcp4 RNA probes, the recombinant hIRP-1 showed a triple band-shift (part B, lanes 3 and 4), as with the control IRE-fer (lane 2); whereas, with the 97-nt deletion mutant tvcp4 mRNA or free RNA transcripts, used as negative controls, no band-shift was observed (lanes 1 and 5). These data show that only tvcp4 RNA probes containing the intact IRE-like structure form RNA–protein complexes with the recombinant hIRP-1.

We then performed UV-induced cross-linking assays with the same 32P-labeled tvcp4 RNA probes and HeLa cytoplasmic extracts, as a source of IRP, to determine the size of the proteins in the RPCs. Fig. 3C shows that 45-kDa and 98-kDa proteins cross-linked with radiolabeled IRE-fer RNA used as a positive control and with the 31- and 94-nt IRE-like tvcp4 RNA probes (lanes 2, 4, and 6, respectively). In addition, a 60-kDa radioactive band was also observed with the trichomonal transcripts (lanes 4 and 6). Neither the 45-kDa, 60-kDa nor 98-kDa proteins cross-linked with the 97-nt transcript of the tvcp4 IRE-like deletion mutant (lane 8). A smaller unknown protein band binding the 97-nt transcript may be an unrelated RNA-binding protein present in HeLa cytoplasmic extracts. As expected, no radiolabeled bands (lanes 1, 3, 5, and 7) were observed in mock experiments. These data show...
that the complete 23-nt stem-loop RNA from tvcp4 is needed for RPC formation with IRP proteins.

3.5. The tvcp4 IRE-like hairpin specifically interacts with an IRP-like protein from HeLa cell extracts

Specificity of RNA–protein binding complexes using the 31-nt IRE-like transcript was demonstrated with UV cross-linking competition assays. We used 10- and 20-fold molar excess of unlabeled homologous 31-nt IRE-like RNA, heterologous IRE-fer RNA, and a non-related tRNA as competitors (Fig. 4A). Unlabeled 31-nt IRE-like RNA partially, up to 79%, and completely competed with homologous labeled RNA (lanes 3 and 8, respectively), as compared to controls without competitor (lanes 2 and 7). On the contrary, unlabeled IRE-fer RNA did not or slightly cross-competed, up to 30%, with the labeled 31-nt IRE-like RNA for specific RPC formation in HeLa cell extracts (lanes 4 and 9). Interestingly, the unlabeled 31-nt transcript, as a heterologous competitor at 10- and 20-fold molar excess (part B), partially, up to 70%, and completely competed with the labeled IRE-fer RNA (lanes 4 and 8) for specific RPC formation, as compared to RPC control (lanes 3 and 7, respectively); whereas, unlabeled homologous IRE-fer RNA partially competed up to 34% and 60% with itself (lanes 5 and 9, respectively), as expected at these molar excesses. An excess of tRNA had no effect on RPC formation with either RNA probes (part A, lanes 5 and 10; part B, lanes 6 and 10). These data show that the tvcp4 RNA and HeLa IRP RNA–protein complexes are specific. These results also show that the tvcp4 IRE-like RNA is a better competitor than the IRE-fer RNA for RPC formation under these experimental conditions. Thus, these data may suggest higher affinity of this unusual IRE-like structure than the IRE-fer RNA for the IRPs present in HeLa cell extracts.

To determine whether the 98-kDa protein observed in the cross-linking experiments using HeLa cell extracts corresponds to the hIRP-1, we performed Western blot assays using...
the anti-recombinant hIRP-1 (α-IRP) antibody. The anti-IRP antibody recognized a 98-kDa band in HeLa cell cytoplasmic extracts (part C, lane 4) and three bands of 123-kDa, 98-kDa, and 60-kDa in the enriched recombinant hIRP-1 protein, which was used as a positive control (lane 3). The anti-GST antibody (used as a control) also recognized the three bands of the recombinant hIRP-1 protein (lane 5), but did not react with the HeLa cells extracts (lane 6). As expected, no protein bands were detected by the pre-immune normal mouse serum (NMS) in any of the tested protein samples (lanes 7 and 8).

We performed immunoprecipitation assays of RPCs with the anti-IRP antibody after the UV cross-linking assays with HeLa cytoplasmic extracts (part D, lane 2). The anti-IRP antibody immunoprecipitated proteins of 98-kDa, 60-kDa, and 45-kDa cross-linked to the 31-nt tvcp4 IRE-like RNA probe (lane 6). Similar immunoprecipitation results were obtained using the RPCs formed with the IRE-fer probe (lane 4) used as a positive control. Pre-immune normal mouse serum (lane 7) and the mock experiment (lanes 3 and 5) with anti-IRP antibody, used as negative controls, did not detect labeled protein bands. These results demonstrate that the 31-nt tvcp4 IRE-like RNA can interact with a 98-kDa protein band, corresponding to the size of HeLa cells IRP-1. The 60-kDa and 45-kDa proteins immunoprecipitated with the anti-IRP antibody may be part of the hIRP-1 degradation products.

3.6. Presence of IRP-like proteins in T. vaginalis and effect of iron on the RPCs formation

To support our hypothesis of gene expression regulation by iron through a post-transcriptional mechanism mediated by an IRE/IRP-like system in trichomonads, we investigated the presence of IRP-like proteins in T. vaginalis cytoplasmic extracts and the effect of iron on RPCs formation. Thus, gel-shifting and cross-linking assays with labeled IRE-fer probe and cytoplasmic extracts of trichomonads grown in iron-rich and iron-depleted medium were performed (Fig. 5). In the gel-shifting assays, two RPC bands were observed with trichomonad extracts from parasites grown in iron-depleted, but not in iron-rich medium (part A, lanes 3 and 4). These complexes showed less mobility than the bands obtained with the rhIRP-1 used as a positive control (lane 2).

We performed UV cross-linking assays using trichomonad cytoplasmic extracts in iron-depleted and iron-rich conditions. A 98-kDa protein cross-linked to radiolabeled IRE-fer RNA was only detected in iron-depleted conditions (part B, lanes 2 and 3). This radioactive band has a similar size to that of the IRP-1 protein from HeLa cells cross-linked to the IRE-fer probe (lane 1), used as a positive control. A 45-kDa radioactive protein was also observed with HeLa extracts (lane 1), probably a degradation product.

4. Discussion

This report shows that synthesis and amounts of the T. vaginalis cysteine proteinase TVCP4 increased in the presence of iron. It appears to be regulated by a post-transcriptional iron regulatory mechanism mediated by specific RNA–protein interactions through an IRE/IRP-like system. This type of regulation in this early-branching protozoan is supported by the presence of an iron-responsive element (tvcp4) at the 5′-end of the TVCP4 mRNA coding region, and cytoplasmic IRP-like proteins in trichomonads. Interestingly, the TVCP4 IRE structure is located in an unusual place for translational regulation. Rather than being in the tvcp4 5′-UTR, it is inside the coding...
This could be due to the short 5'-UTR promoter region in *T. vaginalis* genes [33], leaving the position of this IRE structure within the first 60-nt downstream of the cap structure, which is consistent with the position shown for iron upregulated genes in some mammalian cells [39,40].

Even though a decrease in translational efficiency with IREs located in coding regions has been documented only for artificial constructs thus far [39], it is noteworthy to mention that the IRE in the *tvcp4* mRNA is the first naturally found odd IRE in a gene that is positively regulated by iron at the protein synthesis level. Before this, the only lower organism in which an IRE-binding protein has been identified is *Plasmodium falciparum*, but even in that organism the identification of target mRNAs and post-transcriptional regulation is still somewhat hypothetical [41].

It is also interesting to note that the RNA–protein interactions results and competition data suggest that the *tvcp4* IRE structure has higher affinity for IRP proteins of HeLa cells than the IRE-fer itself. Before this, the only organism in which an IRE-binding protein has been identified is *Plasmodium falciparum*, but even in that organism the identification of target mRNAs and post-transcriptional regulation is still somewhat hypothetical [41].

The IRE interaction is relevant to control translation of mRNA regulated by iron, as occurs with the ferritin gene [14–17]. For *T. vaginalis*, binding of an IRP-like protein to the novel IRE-stem loop structure identified in the *tvcp4* transcript suggests the existence of a post-transcriptional iron regulation mechanism. Our model suggests that at high iron, an IRP-like protein is absent; permitting no IRE/IRP interaction and allowing the *tvcp4* mRNA to be translated for increased amounts of TVCP4 (Fig. 6). This model is consistent with our experimental data and supports the existence of an alternative iron regulatory system in *T. vaginalis*. This is consistent with the insect SDHb, mammalian mitochondrial aconitase, 75-kDa subunit of the mitochondrial complex I, and Alzheimer’s amyloidal precursor protein genes [43–45]. To our knowledge, this is the first time that an IRE/IRP-like iron regulatory system is identified in trichomonads and in protozoa.

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**Fig. 6.** A model to explain the post-transcriptional regulation of the *T. vaginalis* *tvcp4* gene expression by an IRE/IRP-like system. (A) At high-iron concentrations absence of IRP-like proteins (TvIRP) probably due to proteolytic degradation (dashed semicircle) will allow translation of the TVCP4 proteinase. (B) In low-iron concentrations presence of TvIRP-like proteins (semicircle) that bind to the IRE stem-loop structure of the *tvcp4* mRNA will block TVCP4 translation. Thus, TVCP4 will only be observed in trichomonad extracts of parasites grown at high iron concentrations, as shown in Fig. 1D.
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