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Research Paper

The 50 kDa metalloproteinase TvMP50 is a zinc-mediated *Trichomonas vaginalis* virulence factor

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ABSTRACT

Trichomonas vaginalis is a protozoan parasite that can adapt to the trichomonicidal Zn^{2+} concentrations of the male urogenital tract microenvironment. This adaptation is mediated by molecular mechanisms, including proteinase expression, that are regulated by cations such as Zn^{2+}. Herein, we characterized the previously identified 50 kDa metalloproteinase aminopeptidase P (M24 family) member TvMP50 as a new Zn^{2+}-mediated parasite virulence factor. Quantitative RT-PCR and indirect immunofluorescence assays corroborated the positive regulation of both *mp50* gene expression and native TvMP50 protein overexpression in the cytoplasm and secretion products of parasites grown in the presence of Zn^{2+}. Furthermore, this active metalloproteinase was characterized as a new virulence factor by assaying cytotoxicity toward prostatic DU145 cell monolayers as well as the inhibition of parasite and secreted soluble protein proteolytic activity in the 50 kDa proteolytic region by the specific metalloproteinase inhibitor 1,10-phenanthroline and the chelating agents EDTA and EGTA. Parasite and secreted soluble protein cytotoxicity toward DU145 cells were reduced by treatment with an α-rTvMP50 polyclonal antibody. Our results show that the metalloproteinase TvMP50 is a new virulence factor modulated by Zn^{2+}, which is present during male trichomoniasis, possibly explaining *T. vaginalis* survival even within the adverse conditions of the male urogenital microenvironment.

1. Introduction

Trichomonas vaginalis is an extracellular protozoan parasite that causes an estimated 276 million annual cases of human trichomoniasis [1,2]. This parasite infects the human urogenital tract [3] and increases the predisposition to HIV infection and the development of cervical cancer [4]. As trichomoniasis is asymptomatic in the majority of cases in men [5], men are considered to be a reservoir [6,7]; however, infection is associated with a predisposition to the development of prostate cancer [8]. *T. vaginalis* can adapt and colonize prostatic tissue even under the adverse microenvironmental conditions presented by prostatic fluid Zn^{2+} concentrations (4–7 mM) [9–11]. The minimal trichomonicidal concentration (MTC) of Zn^{2+} is 1.6 mM, and prostatitis with parasitic infection has been found in patients with Zn^{2+} below the MTC [10–12]. The function of Zn^{2+} is primarily related to the innate immune responses in the male urogenital tract, that hinder pathogen development [13]. Male trichomoniasis has been little studied, and the pathogenic mechanisms of *T. vaginalis* and factors leading to disease are

Abbreviations: BFS, bovine fetal serum; CDS, coding DNA sequence; CPs, cysteine proteinases; HIHS, heat inactivated horse serum; *mp50*, gene encoding the 50 kDa metalloproteinase; MS, mass spectrometry; nt, nucleotide; NMS, mouse preimmune serum; TCA, trichloroacetic acid; TvMP50, 50 kDa metalloproteinase of *Trichomonas vaginalis*; TYM, trypticase–yeast extract–maltose; WB, western blot; α-rTvDHS, rabbit anti-recombinant DHS antibody; α-rTvMP50, mouse anti-recombinant TvMP50 polyclonal antibody

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unknown [14]. Proteinases that have been identified as factors contributing to infection maintenance, *e.g.*, the TvCP65 and TvCP39 cysteine proteinases (CPs), are involved in cervical cell cytotoxicity [15–17]. These CPs are modulated by microenvironmental conditions such as the presence of cations (*e.g.*, Fe^{2+} and Zn^{2+}), polyamines [18] and oxygen stress [19]. The *T. vaginalis* genome contains 446 genes that encode proteinases, with CPs and metalloproteinases (MPs) being the most abundant [20]. The MPs of family M24 subfamily B have three histidine residues that are required to coordinate a Zn^{2+} ion within the catalytic site for substrate proteolysis [21]. The aminopeptidase P (M24) family can also bind Mn^{2+} [22]. In parasites, several MPs families that are not in the clan MG family M24 have functions that can be classified as virulence factors. For example, MP falcilysin, participates in the degradation of human red blood cells facilitating the infection by *Plasmodium falciparum* [23], and the GP63-like MP localized on the surface of *Trypanosoma cruzi* [24] and *Leishmania major* modulates host-infections [25,26]. In the case of *T. vaginalis,* cytotoxicity toward prostatic and cervical cells is negatively affected by parasite growth in the presence of Zn^{2+} [27]. Recently, the homodimeric hydrogenosome processing protease (HPP) MP was characterized and found in the *T. vaginalis* hydrogenosome [28]. This parasite also expresses a GP63-like metalloproteinase, TvGP63, which is localized to the parasite membrane and involved in the cytotoxicity of *T. vaginalis* toward HeLa cell monolayers [29]. Moreover, the presence of MPs in *T. vaginalis* excretion and secretion products induces a host cell response [30]. Recently, we reported that TvMP50 is an immunogenic MP that could be present and active during male trichomoniasis because it is recognized by sera from male but not female trichomoniasis patients and that Zn^{2+} promotes native TvMP50 proteolytic activity [31]. This work aims to characterize the function of TvMP50 as a new *T. vaginalis* virulence factor. TvMP50 activity is mediated by Zn^{2+}, the main cation present during interaction with prostatic cells in the male urogenital microenvironment. We demonstrate the effects of Zn^{2+} on the expression of the *mp50* gene and TvMP50, as well as the subcellular localization and proteolytic activity of secretion products and cytotoxicity toward DU145 cells.

2. Materials and methods

2.1. Parasite and DU145 cell culture

Male isolate *T. vaginalis* HGMN01 and female isolate CNCD147 parasites were grown to mid-logarithmic phase in Diamond's trypticase-yeast extract-maltose (TYM) medium (pH 6.2) supplemented with 10% (v/v) heat-inactivated horse serum [32]. The final Zn^{2+} concentration (Sigma-Aldrich, Co., St. Louis, MO, USA) in all assays was 1.6 mM [27]. A prostatic cell line, DU145, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) and ampicillin/streptomycin (100 μg/ml) with 5% CO_2 at 37 °C.

2.2. Analysis of the mp50 gene sequence

Analysis of the 5′ region of the *mp50* gene was performed using sequences obtained from the *T. vaginalis* database TrichDB (http://trichdb.org/trichdb) and the reported ESTs from the previously reported Clan MG, family M24, aminopeptidase P-like metallopeptidase (accession number TVAG_403460) [31].

2.3. RNA isolation and quantitative real time PCR

The total RNA was extracted from 10×10^6 parasites grown with or without Zn^{2+} using TRIzol® (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. For qRT-PCR, total RNA (1 μg) was reverse transcribed using a Superscript II reverse transcriptase kit (Invitrogen) and oligo (dT20) primer. Reactions contained one microgram of cDNA from each condition as template, 10 pmol of each primer specific to the *mp50* gene forward primer (5′-CGACTGGGACTGGGCTTTGAAACTCG-3′) and reverse primer (5′-TCAAAGTACTCTTGAAGC-3′) and 2× SYBR Green I PCR Master Mix (Applied Biosystems, Waltham, MA, USA). As a control, a 112 bp region of the β-tubulin gene was amplified using the BTUB9 forward primer (5′-CATTGATAACGAAGCTCCTTTACGAT-3′) and the BTUB2 reverse primer (5′-GCATGTTGTGCCGGACATAACCAT-3′) [33]. The cycling conditions were 10 min of polymerase activation at 95 °C followed by 35 cycles at 95 °C for 30 s, 55 °C for 15 s and 72 °C for 30 s. β-tubulin mRNA levels were used for normalization and each assay was performed twice in triplicate.

2.4. Immunolocalization of T. vaginalis TvMP50

For indirect immunofluorescence assays, parasites of male isolate *T. vaginalis* HGMN01 and female isolate CNCD147 were grown on sterile coverslips with and without Zn^{2+} (Sigma), washed with filtered 1× PBS (pH 7.0) and fixed with 4% paraformaldehyde for 1 h at 37 °C. The fixed trichomonads were then permeabilized with 0.2% Triton X-100 for 15 min at 25 °C, washed twice with filtered 1× PBS (pH 7.0) and blocked with 0.2 M glycine for 1 h at 37 °C and 0.2% fetal bovine serum for 15 min at 25 °C. The slides were then incubated with mouse anti recombinant TvMP50 antibody (α-rTvMP50) serum (dilution 1:150) overnight at 4 °C, washed three times with 1× PBS (pH 7.0), incubated with fluorescein-isothiocyanate(FITC) conjugated goat anti-mouse immunoglobulin (diluted 1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at 25 °C, and washed three times with 1× PBS (pH 7.0). The slides were then mounted using Vectashield-DAPI solution (Vector Laboratories, Burlingame, CA, USA) to label nucleic acids, and then observed by confocal microscopy (Leica Microsystems, Wetzlar, Germany).

2.5. In vitro secretion assays

Male isolate *T. vaginalis* HGMN01 and female isolate CNCD147 parasites were washed three times with 1× PBS/0.5% maltose and resuspended in the same solution (1×10^6 parasites/ml). Parasite viability was measured by trypan blue exclusion (Sigma). The parasites were incubated at 37 °C for 3 and 6 h and then centrifuged at 495 × *g*. The supernatant was clarified by centrifugation at 13,000 × *g* and filtered through a 0.22 μm membrane to obtain the soluble secreted proteins. One aliquot was stored at −80 °C until used in zymogram and cytotoxicity assays, and another was concentrated by precipitation with 10% TCA for SDS-PAGE and WB analysis using purified polyclonal α-rTvMP50 antibody [31] and polyclonal α-rTvDHS (deoxyhypusine synthase/hydrolase) as a negative control for secretion [33].

2.6. Zymogram assays for rTvMP50, live parasites and T. vaginalis soluble secreted proteins

For proteolytic activity assays, we incubated 5.0 μg of rTvMP50 with or without the chelating agents EDTA, EGTA or 1,10-phenantrholine (1,10-PT) (5 mM final concentration) for 20 min at 4 °C [33]. Parasites (20×10^6) were grown with or without Zn^{2+} and then incubated 30 min with or without EDTA, EGTA and 1,10-PT chelants. The parasites were then washed three times with 1× PBS (pH 7.0) and extracts were loaded onto substrate gels under the same conditions used above. Samples of 6 h of secretion products (100 μg) were treated with 5 mM EDTA, EGTA and 1,10-PT for 20 min at 4 °C, and 0.25% Coomassie brilliant blue-stained gels showed proteolytic activity as white bands on a dark background. A representative image of three gels was captured with a ChemiDoc MP System using Image Lab software (version 4.0; Bio-Rad, Hercules, CA, USA).

2.7. In gel digestion of protein band in zymogram

The sections of zymogram gels were dissected manually and destained with 2.5 mM NH_4HCO_3 in 50% acetonitrile (ACN) and then dehydrated with 100 µl of 100% ACN. The proteins were reduced with 20 µl of 10 mM DTT in 50 mM NH_4HCO_3 and incubated for 45 min at 56 °C. After that, samples were cooled down at 25 °C and alkylated with 100 mM iodoacetamide in 50 mM NH_4HCO_3 and incubated in the dark for 30 min. Gel cubes were washed with 100 µl of 100% ACN for 5 min. After the solution removal, the gel cubes were washed with 100 µl of 5 mM NH_4HCO_3 for 5 min. Then, the samples were washed again with 100 µl of 100% ACN for 5 min. Finally, the gel cubes were dried with a CentriVap (Labconco, Kansas, MO, USA) for 5 min.Then, the samples were rehydrated with 10 µl of digestion solution containing 12.5 ng/µl mass spectrometry grade Trypsin Gold (Promega, Madison, WI, USA) in 5 mM NH_4HCO_3. The reaction was carried out in a water bath at 37 °C overnight and stopped at −80 °C. The resulted peptides were extracted three times with 30 µl of 50% acetonitrile with 5% formic acid by centrifugation at 1000 × *g* for 30 s. Finally, samples were desalted with ZipTip-µC18 tips (Merck Millipore, Darmstadt, Germany) and dried using a CentriVap (Labconco).

2.8. Nano LC–MS/MS analysis

Samples constituted with 5 µl of 0.1% formin acid were injected into a nanoviper C18 trap column (3 µm, 75 µm × 2 cm, Dionex, Sunnyvale, CA, USA) at a 3 µl min^{-1} flow rate, and then separated on an EASY Spray C18 RSLC column (2 µm, 75 µm × 25 cm) with a flow rate of 300 µl min^{-1} connected to an UltiMate 3000 RSLC system (Dionex) and interfaced with an OrbitrapFusoin™ Tribid™ (Thermo-Fisher Scientific, San Jose, CA. USA) mass spectrometer equipped with an "EASY Spray" nano ion source (Thermo-Fisher Scientific). For peptide separation, a chromatographic gradient using MS grade water with 0.1% formic acid (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B) for 30 min was set as followed: 10 min solvent A, 5–25% solvent B within 20 min, 25–95% solvent B for 5 min, 95% solvent B for 10 min and 95–5% solvent B for 5 min. The mass spectrometer was operated in positive ion mode with nanospray voltage set at 3.5 kV and source temperature at 280 °C. External calibrants included caffeine, Met-Arg-Phe-Ala (MRFA) and Ultramark 1621. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS. Briefly, a survey of full-scan MS spectra were acquired in the Orbitrap analyzer, scanning of mass range was set to 350–1500 *m/z* at a resolution of 120,000 (FWHM) using an automatic gain control (AGC) setting it to 4.0e5 ions, setting the maximum injection time to 50 ms, and using dynamic exclusion 1 at 90 s and 10 ppm mass tolerance. Afterwards, a top speed survey scan for 3 s was selected for subsequent decision tree-based Orbitrap *collision-induced dissociation* (CID) or higher-energy collisional dissociation (*HCD*) fragmentation [34,35]. The signal threshold for triggering an MS/MS event was set to 1.0e4 and the normalized collision energy was set to 35% and 30% for CID and HCD, respectively. The AGC of 3.0e4 and isolation window of 1.6 *m/z* was set for both fragmentations. Additional parameters for CID included activation Q which was set to 0.25 ms with an injection time of 50 ms. For HCD, first mass was set to 120 *m/z* and injection time to 100 ms. The settings for the decision tree were as follows: For CID fragmentation charge states 2 or 3 were scanned in a range of 650–1200 *m/z*. For HCD fragmentation, charge state 3 was scanned in a range of 300–6500 *m/z*, charge state 4 were scanned in a range of 300–900 *m/z*, and charge state 5 had a scan range of 300–950 *m/z*. All data were gathered with Xcalibur 4.0.27.10 software (Thermo-Fisher Scientific).

2.9. Data analysis and interpretation

The raw data were processed with Proteome Discoverer 2.1 (PD, Thermo Fisher Scientific Inc.). The subsequent searches were carried out using Mascot server version 2.4.1 (Matrix Science, Boston, MA) and SQUEST HT [36]. The search with both engines was concerned with *Trichomonas vaginalis* reference proteome (http://www.uniprot.org/proteomes/UP000001542). The search parameters included full-tryptic protease specificity, two missed cleavage allowed and static modifications covered carbamidomethylation of cysteine (+57.021 Da). Furthermore, the dynamic modifications included methionine oxidation (+15.995 Da) and deamidation in asparagine/glutamine (+0.984 Da). For the MS2 method, identification was performed at high resolution in the Orbitrap, where precursor and fragment ion tolerances of ± 10 ppm and ± 0.2 Da were used. The resulting peptide hits were filtered for a maximum of 1% FDR using the percolator algorithm [37].

2.10. Cytotoxicity assays using endogenous T. vaginalis TvMP50

DU145 cell monolayers (4×10^4 cells/well) were grown in 96-well plates and then incubated with 20×10^5 of male clinical isolate *T. vaginalis* HGMN01 parasites (5:1 parasite-host cell ratio) grown with and without Zn^{2+}. Other plates were treated with and without 5 mM EDTA, EGTA and 1,10-PT in interaction media (DMEM and TYM medium 2:1 v/v) without IHHS for 6 h at 37 °C in a 5% CO_2 atmosphere. The effect of mouse anti α-rTvMP50 polyclonal antibody on cytotoxicity was also determined using 0, 50 and 75 µg/ml of purified antibody or purified IgG from normal mouse serum (NMS) as negative control as previously described. Damage to the DU145 monolayer was quantified using a colorimetric assay [15]. To visualize secreted protein-induced cytotoxicity, DU145 monolayers in six-well plates (20×10^4 cells/well) were incubated with 200 or 400 µg of 6 h secretions from male isolate (HGMN01) grown with or without Zn^{2+} in PBS-0.5% maltose and 400 µg of secretions of female (CNCD147) isolate parasites grown with Zn^{2+} in PBS-0.5% maltose were incubated 3 and 6 h with DU145 cell monolayer (20×10^4 cells/well) for cytotoxic profile comparisons between isolates. The cells were washed three times with sterile 1× PBS (pH 7.2), fixed with 2% formaldehyde and stained with 0.13% crystal violet. Quantitative assays performed twice in triplicate produced similar results and representative images of cytotoxicity were captured with a ChemiDoc MP System using Image Lab software (version 4.0; Bio-Rad).

2.11. Cytotoxicity assays of recombinant T. vaginalis TvMP50

The cytotoxicity of rTvMP50 toward prostatic cells was tested similarly to our determination of native TvMP50 by treating DU145 monolayers with 1.0 or 5.0 µg/ml of recombinant protein and visualized using a colorimetric assay with rTvDHS [38] used as a negative control, and DU145 monolayers without parasites were used as a control for monolayer integrity. The visual effects of cytotoxicity were also determined with or without the addition of 5 mM EDTA, EGTA and 1,10-PT. Two independent quantitative assays performed in triplicate produced similar results, and representative images of visual cytotoxicity were captured with a ChemiDoc MP System using Image Lab software (version 4.0; Bio-Rad).

2.12. Statistical analysis

All data are expressed as the mean ± S.D. of three samples. All experiments were performed twice in triplicate. The significance of the difference between the means was determined by ANOVA using GraphPad Prism 5.0. The level of significance was also determined using the Bonferroni multiple comparisons test.

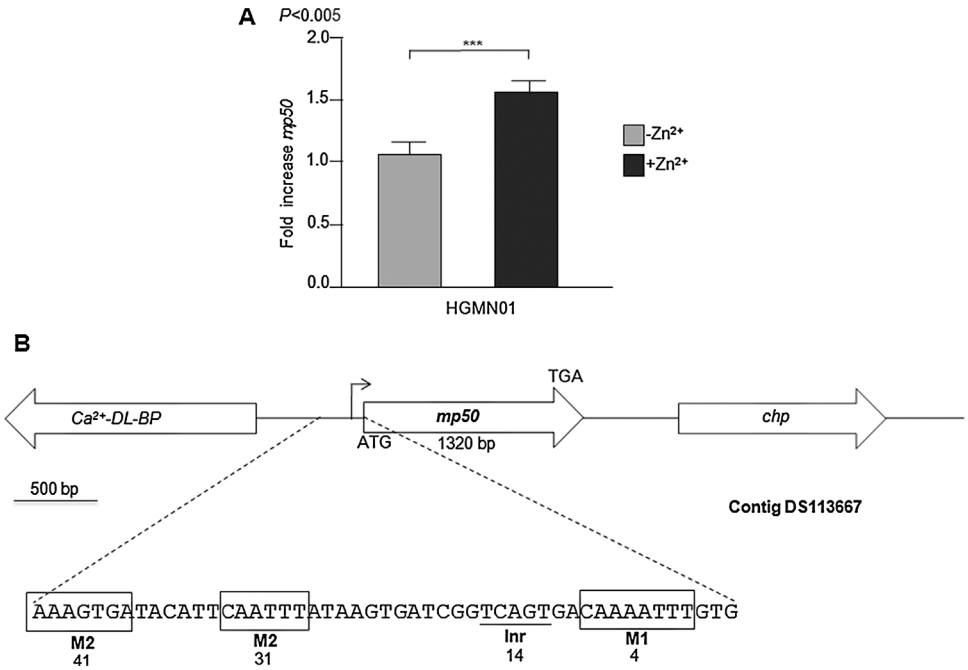

Fig. 1. Expression and genic organization of *mp50* in *T. vaginalis* HGM01 isolate. (A) Quantitative RT-PCR assays with specific primers for *mp50* gene (TVAG_403460) showing the gene expression upregulation with Zn^{2+} 1.6 mM. β-Tubulin gene fragment was used to normalize the expression levels. Two independent assays were performed in triplicate with similar results. The error bars indicate the standard deviation (SD). A significant difference ($p < 0.005$) was found in fold-increase in the expression of *mp50* gene with Zn^{2+} marked with three asterisks compared with the control condition (B) The possible 5'-UTR region of *mp50* genomic sequence was analyzed showing a putative initiator promoter element (Inr) (curved arrow and underlined nucleotide sequence), and the box indicate the position of the possible M1 and M2 motif elements. Bar represents 500 bp.

3. Results

3.1. Zn^{2+} upregulates mp50 gene expression

Previously, the gene and protein sequences of TvMP50 (TVAG_403460) were found to encode a metalloproteinase aminopeptidase-P member of the clan MG family M24, subfamily B. The catalytic amino acids H215, H324, H335, D232, D243, H328, Q364, and Q407 serve as metal ion ligands (Fig. S1). Despite its identification in *T. vaginalis* as an immunogenic, active metalloproteinase in the degradome of parasites grown with 1.6 mM of Zn^{2+} [31], the mechanisms underlying *mp50* gene expression were unknown. qRT-PCR assays were performed to examine *mp50* gene expression quantitatively and showed that *mp50* mRNA changed 1.5-fold in parasites grown in with Zn^{2+} compared with parasites grown without Zn^{2+} conditions when normalized using a 112 bp region of the β-tubulin gene fragment (Fig. 1A). Although positive Zn^{2+}-mediated regulation of this metalloproteinase gene was now demonstrated, we sought to identify potential basal Inr promoter elements [39] Two typical regulatory motifs 2 (M2) were found 41 nt present in this parasite [40] and an other M2 was found 31 nt upstream of the ATG initiation codon. An additional motif 1 (M1) was also found 4 nt upstream of the ATG initiation codon [40]. *In silico* analysis of the *mp50* gene revealed a possible Inr promoter 14 nt upstream of the ATG initiation codon, located in putative 5′-UTR. The ESTs analysis of paralogue genes of MG clan for *mp50 T. vaginalis* showed 13 ESTs under different growth conditions (Fig. S2). However, no ESTs have been reported in Zn^{2+} conditions, yet. The reported ESTs for TvMP50 did not permit the identification of the putative transcriptional start site (TSS) as all sequences were contained within the gene ORF (Fig. 1B).

3.2. Zn^{2+} also promotesTvMP50 expression and localization in T. vaginalis

To determine the subcellular localization of TvMP50 in the *T. vaginalis* male HGMN01 isolate, we performed indirect immunofluorescence assays using polyclonal α-rTvMP50 mouse sera and fixed parasites grown with or without Zn^{2+}. Strong fluorescence intensity (Fig. 2A, panel b, green) was observed in vesicle-like structures in the cytoplasm of parasites grown with 1.6 mM Zn^{2+} (Fig. 2A, panel b, white arrows) while parasites grown without Zn^{2+} had less fluorescence intensity and vesicle-like structures were not observed (Fig. 2B, panel d). A lower signal intensity was localized within the female CNCD 147 isolate parasites even in Zn^{2+} conditions (Fig. S4) which contrasted with the intense fluorescent signal observed in vesicular-like structures in the parasites of HGMN01 isolate in Zn^{2+} (Fig. 2A, panel b, white arrows). The presence of TvMP50 in these structures suggests a different localization from that in with the metalloproteinase TvGP63 (TVAG_367130), which has been previously reported to be primarily localized on the *T. vaginalis* surface [29].

3.3. Metalloproteinase TvMP50 is secreted by T. vaginalis with or without Zn^{2+} conditions

The *T. vaginalis* parasite has been shown to secrete several molecules into the media, some of which act as virulence factors and are modulated by host and environmental conditions, *e.g.*, cell-detaching factor (CDF), exosomes and CPs (reviewed in [41]) and the metalloproteinase TvGP63 [29]. To investigate whether the TvMP50 metalloproteinase is also secreted by the parasite, we performed *in vitro* secretion kinetics assays in PBS-maltose for 3 and 6 h using parasites that were grown with or without Zn^{2+} that presented 95% viability as per by trypan blue exclusion tests. WB assays of TCA-precipitated media containing parasite secretions with α-rTvMP50 detected a 50 kDa band corresponding to this metalloproteinase among the secreted proteins. Interestingly, less TvMP50 was detected in the media following a 6 h incubation in PBS-maltose for parasites grown with 1.6 mM Zn^{2+} (Fig. 3B). The α-rTvDHS antibody was used to detect DHS, an expected cytoplasmic protein [31], as a negative secretion protein control. Consistent with this expectation, TvDHS was only detected in parasites total extract (Fig. 3B). To confirm the active secretion of TvMP50, we used the α-rTvCP39 antibody to detect secreted TvCP39 as a positive control (data not shown); additionally, TvCP39 has been described as a virulence factor [14,39]. These results indicate that TvMP50 is secreted by living *T. vaginalis* but that Zn^{2+} downregulates the amount of secreted protein.

3.4. The TvMP50 present in T. vaginalis secretions is an active metalloproteinase

Gelatin zymograms of extracts from parasites grown with or without

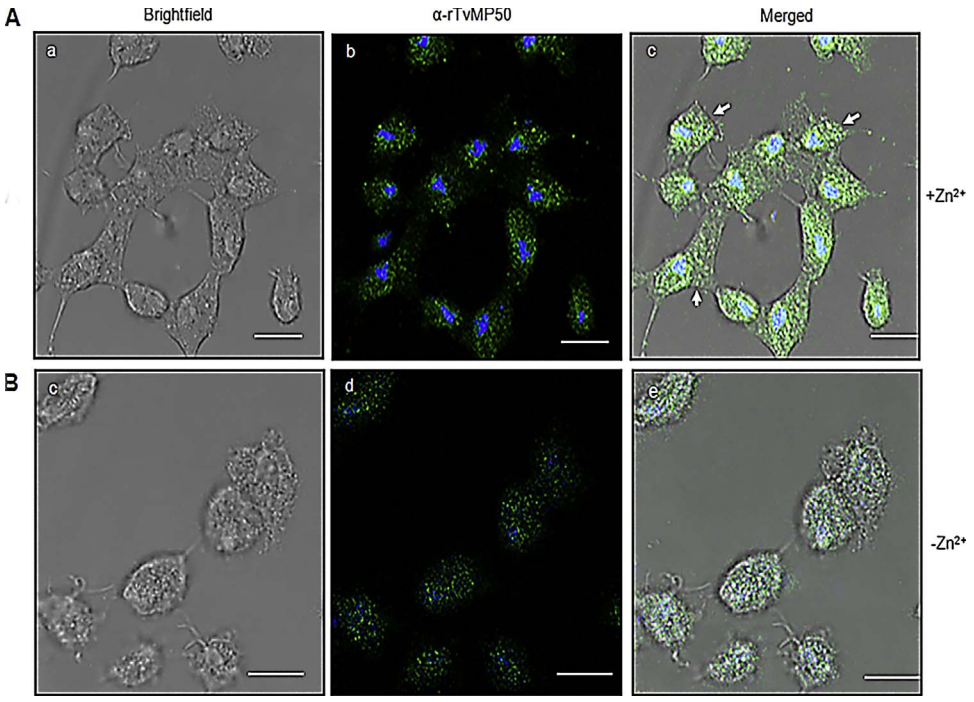

Fig. 2. Localization of TvMP50 in *T. vaginalis* HGMN01 isolate. (A) Identification of TvMP50 in parasites grown with zinc ($+Zn^{2+}$) in cytoplasm with α-rTvMP50 followed by FITC-conjugated goat anti-mouse IgG (panel b, green) and vesicle-like structures (panel c, green, white arrows merged with brightfield, a). (B) Identification of TvMP50 in parasites grown without zinc ($-Zn^{2+}$) and in the cytoplasm with α-rTvMP50 followed by FITC-conjugated goat anti-mouse IgG (panel d, green). Non vesicle-like structures were found (panel e, merged with brightfield c). Nuclei stained with DAPI (blue). The slides were observed at 63× magnification by confocal microscopy (Leica). Scale bar indicates 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Zn^{2+} show the parasite proteolytic profile under these conditions. In this analysis, prominent proteolytic bands were observed in the 30, 39 and 65 kDa regions (Fig. 4A, lane 1), as well as in the 50 kDa region (Fig. 4A, lane 3, white asterisks). To determine whether the protein present in the 50 kDa proteolytic region was TvMP50, we pre-treated the parasites with 5 mM chelating agents EDTA [31], EGTA and the inhibitor 1,10-phenanthroline (1,10-PT), which affect the 71 kDa TvGP63 metalloproteinase gelatinase activity [30]. We observed a reduction in the proteolytic bands of extracts from parasites grown without Zn^{2+} conditions near 50 and 71 kDa upon treatment with EDTA and EGTA, and an almost total loss of proteolytic activity in the same regions in extracts from parasites treated with 1,10-PT (Fig. 4A, dark asterisks), demonstrating the effects of these treatments on MP activity. Only the 30 kDa region band retained proteolytic activity. For *T. vaginalis* grown with 1.6 mM Zn^{2+}, the total proteolytic activity was increased (Fig. 4A), and parasites treated with 5 mM EDTA, EGTA and 1,10-PT retained activity of not only the main proteinases but also the 71 kDa (Fig. 4A, dotted arrow) and 50 kDa regions (Fig. 4A, dark full arrow). To determine whether TvMP50 was also active in parasite secretions, secretions from parasites grown with or without Zn^{2+} incubated in PBS-maltose under the same Zn^{2+} conditions were obtained. The total proteolytic profiles of the secretions were reduced in without Zn^{2+} conditions (Fig. 4B) compared with the total extract (Fig. 4A, lane 1). Interestingly, treatment with EDTA and EGTA revealed increased proteolytic activity of higher molecular weight proteinases in the secretions, while treatment with 1,10-PT eliminated secreted proteolytic activity (Fig. 4A and B, $-Zn^{2+}$). Parasites grown with 1.6 mM Zn^{2+} in the PBS-maltose medium (Fig. 4B, $+Zn^{2+}$ lane 1) also exhibited reduced secreted proteolytic activity compared with the total extract (Fig. 4A, $+Zn^{2+}$ lane 1); however, secretions and total extracts had more proteolytic activity in with Zn^{2+} conditions. Treatment with EDTA reduced the activity of interest in the metalloproteinase region (50 kDa) less than the EGTA treatment (Fig. 4B, $+Zn^{2+}$). The treatment with 1,10-PT did not inhibit the secreted proteolytic activity in with Zn^{2+} conditions but it inhibited the 71 kDa proteolytic region, (Fig. 4B, $+Zn^{2+}$, dotted arrow) that suggest the presence of this metalloproteinase also in the secretions and TvMP50 (Fig. 4A, $+Zn^{2+}$, dark full arrow) regions as well. In order to confirm this data, we performed Nano LC–MS/MS analysis of the proteolytic secretions of the 50 kDa region that allowed for the identification of the TvMP50 (TVAG_403460, access number A2F8Y2, Supp. Table S1) with high confidence with 17 peptides (Table 1, Supp. Table S1). Three of these peptides were divergent with respect to the paralogue metalloproteinase (TVAG_ 040850), (Fig. S3). This sequence had 71% of identity with respect to TvMP50 (TVAG_403460).

3.5. The endogenous and secreted metalloproteinase TvMP50 has cytotoxic activity toward DU145 cells

To examine the biological effects of endogenously secreted TvMP50

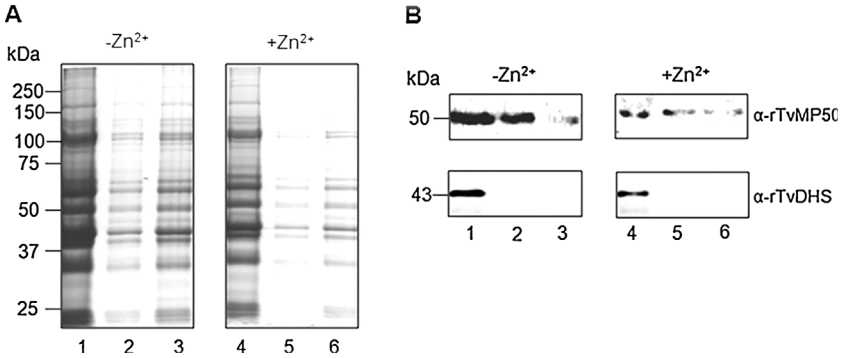

Fig. 3. TvMP50 is a secreted protein of *T. vaginalis* HGM01 isolate. (A) The CBB profile of TCA 10% precipitated secreted proteins of parasites without ($-Zn^{2+}$) and with zinc ($+Zn^{2+}$) after 3 h (lanes 2 and 5) and 6 h (lanes 3 and 6) at 37 °C in PBS-maltose 0.5%. Total protein extracts in both conditions are shown for both conditions (lanes 1 and 4). (B) Identification of TvMP50 in secretions using α-rTvMP50 antibody of parasites without ($-Zn^{2+}$) and with zinc ($+Zn^{2+}$) at 3 h (lanes 2 and 5) and 6 h (lanes 3 and 6). Recognition of TvMP50 in total extract in both conditions (lanes 1 and 4). The α-rTvDHS antibody was used as negative secretion control only detected in parasite TE in both conditions (lane 1 and 4) kDa. Molecular weight marker (Prestained SDS-PAGE Standards, Bio-Rad) in kilodaltons.

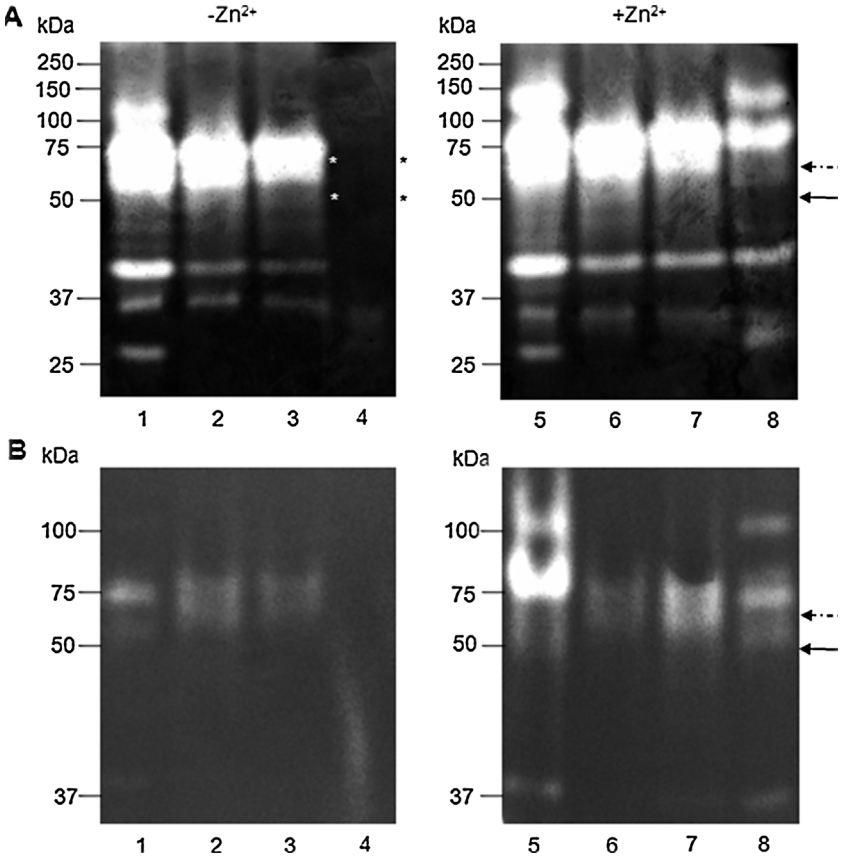

Fig. 4. Endogenous and secreted TvMP50 of *T. vaginalis* is proteolytic active. (A) Zymogram showing the proteolytic profile of TE of *T. vaginalis* HGM01 grown without ($-Zn^{2+}$) (lane 1) or with zinc ($+Zn^{2+}$) (lane 5) conditions and treated with EDTA (lanes 2 and 6), EGTA (lane 3 and 7) or 1,10-PT (lane 4 and 8). (B) Zymogram showing the proteolytic profile of secreted proteins of *T. vaginalis* HGM01) grown without ($-Zn^{2+}$) or with zinc ($+Zn^{2+}$) conditions (lanes 1 and 3) and treated with EDTA (lanes 2 and 6), EGTA (lanes 3 and 7) or 1,10-PT (lane 4 and 8). The secretions were obtained of parasites incubated 6 h at 37 °C in PBS-maltose 0.5%. The 50 kDa protein proteolytic activity band (Full arrow) was processed for nano LC–MS/MS identification. kDa. Molecular weight marker (Prestained SDS-PAGE Standards, Bio-Rad) in kilodaltons.

Table 1
Nano LC–MS/MS information on the identification of A2F8Y2 (TvMP50).

Confidence	Sequence	Modifications	MPA[a]
High	**NLIEVFKQNSIDHGIIVFK**		A2F8Y2
High	QIELCNMIKPGLTFAEMNK	1xCarbamidomethyl [C5]	A2F8Y2
High	**TCRPGNIVTI**EPGLYFHK	1xCarbamidomethyl [C2]	A2F8Y2
High	**YMYQCLEAIGLIKK**	1xCarbamidomethyl [C5]	A2F8Y2
High	FIDDLKHDIQGK		A2F8Y2
High	EKSFLTIAASGQDAVYLHNSANEGVCK	1xCarbamidomethyl [C26]	A2F8Y2
High	KAAELTSEAIIHVMK	1xOxidation [M14]	A2F8Y2
High	KAAELTSEAIIHVMK		A2F8Y2
High	QNSIDHGIIVFK		A2F8Y2
High	AKTPYEIEQIKK		A2F8Y2
High	MHDQTALPSAAGIAR		A2F8Y2
High	IEDDMLVTETGNQR		A2F8Y2
High	**SDLIKDTNDEAR**		A2F8Y2
High	**EMTVDEKNQNEIAR**		A2F8Y2
High	AAELTSEAIIHVMK	1xOxidation [M13]	A2F8Y2
High	AAELTSEAIIHVMK		A2F8Y2
High	NIKPGWSEQQVDAEFTYYGFK		A2F8Y2
High	SFLTIAASGQDAVYLHNSANEGVCK	1xCarbamidomethyl [C24]	A2F8Y2
High	LADEVGGIRIEDDMLVTETGNQR	1xOxidation [M14]	A2F8Y2
High	LADEVGGIRIEDDMLVTETGNQR		A2F8Y2

[a] MPA: Master Protein Accesions.

on host cells, we analyzed the effects of rTvMP50 on the integrity of DU145 monolayers. After 1 h of incubation, 1.0 μM of rTvMP50 disrupted 30% of the monolayer and it had disrupted 55% of the monolayer was disrupted 6 h compared with the non-metalloproteinase rTvDHS, which had no cytotoxic effects compared with the integrity of the monolayer without recombinant protein (Fig. 5A). Furthermore, to observe the effects of secreted TvMP50, 200 and 400 μg of total secreted proteins from parasites grown with or without Zn^{2+} were co-incubated with DU145 monolayers, and cytotoxic activity was visually determined by colorimetric assay. Monolayer disruption was observed for both protein concentrations for the without Zn^{2+} treatment at 6 h (Fig. 5B). For conditions with and without Zn^{2+} a more evident cell monolayer was observed at the same concentrations and incubation time (Fig. 5C).

Once we had determined the cytotoxic effect of rTvMP50, we evaluated the effects of native *T. vaginalis* TvMP50 as a new endogenous virulence factor against the host, as well as whether the proteinase is present in Zn^{2+} secretions. To confirm this, parasites grown in sterile-PBS with or without Zn^{2+} were treated with 50 or 75 μg/ml of purified α-rTvMP50 antibody and then co-incubated with DU145 monolayers in

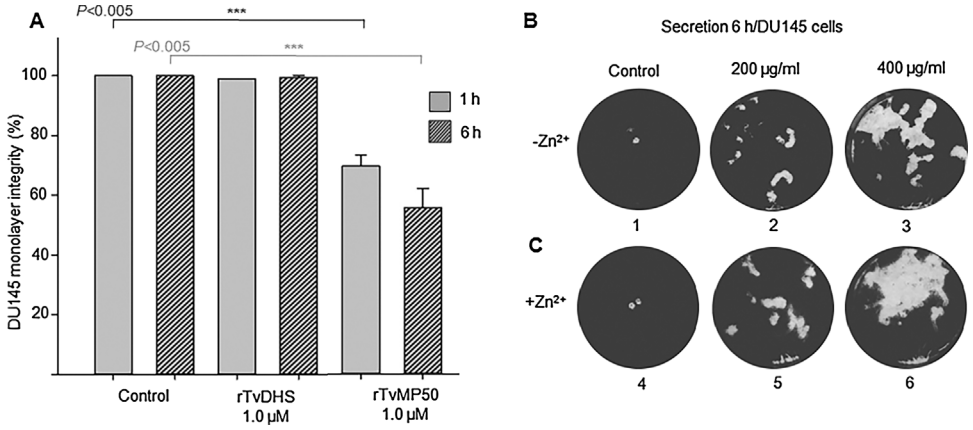

Fig. 5. rTvMP50 and secretions of *T. vaginalis* are cytotoxic to DU145 cells. (A) Quantification of cytotoxic effect of rTvMP50 over the DU145 monolayer integrity showed as 100% at 1 and 6 h of incubation (B) The visual cytotoxic effect of 200 µg/ml (circle 2) and 400 µg/ml (circle 3) of secreted proteins of *T. vaginalis* HGM01 without zinc ($-Zn^{2+}$) conditions. (C) Visual cytotoxic assay showing the effect of 200 µg/ml (circle 5) and 400 µg/ml (circle 6) of secreted proteins of *T. vaginalis* HGM01 with zinc ($+Zn^{2+}$). The integrity of the confluent monolayer of DU145 cells was fixed and stained with crystal violet (circles 1 and 4). Two independent assays in triplicate were performed, and the error bars indicate the SD. A significant difference ($P < 0.005$) was found for rTvMP50 treatment marked with three asterisks when compared with the control condition.

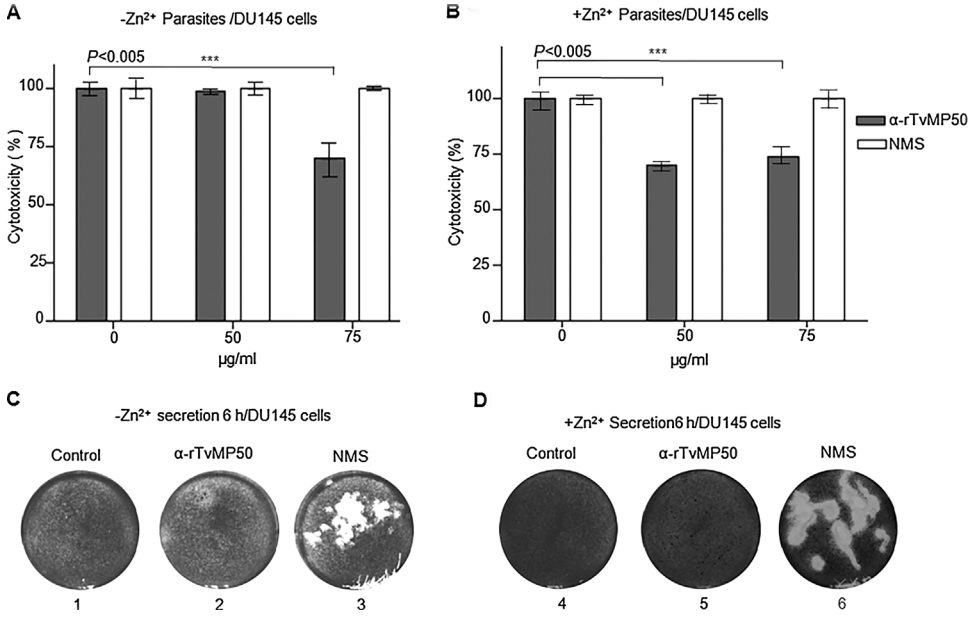

Fig. 6. Endogenous and TvMP50 in secretions of *T. vaginalis* are cytotoxic to DU145 cells. (A) Quantification of cellular damage of parasites without zinc ($-Zn^{2+}$) co-incubated with DU145 monolayer showed 100% of cytotoxicity and parasites treated with α-rTvMP50 (grey bars) or NMS (white bars) at 6 h of incubation. (B) Quantification of cellular damage of parasites with zinc ($+Zn^{2+}$) co-incubated with DU145 monolayer showed 100% of cytotoxicity and treated with α-rTvMP50 (grey bars) or NMS at 6 h of incubation (white bars) Two independent assays in triplicate were performed, and the error bars indicate the SD. A significant difference ($P < 0.005$) was found for α-rTvMP50 treatment compared with the control condition is marked with three asterisks. (C) A representative image of the visual reduced cytotoxicity of 400 µg/ml of secreted proteins without zinc ($-Zn^{2+}$) treated with 75 µg of α-rTvMP50 (circle 2) or with NMS (circle 3). (D) A representative image of the visual reduced cytotoxicity of 400 µg/ml of secreted proteins with zinc ($+Zn^{2+}$) treated with 75 µg of α-rTvMP50 (circle 5) or with NMS (circle 6). Non-treated DU145 cells monolayer fixed and crystal violet stained was used as monolayer Integrity control (circles 1 and 4).

interaction media with or without Zn^{2+} (TYM:DMEM, 1:2 v/v). After six hours of incubation, cytotoxic activity was evaluated by plate colorimetric assays and observed to be reduced 25% following treatment with 75 µg/ml of antibody for parasites grown without Zn^{2+} (Fig. 6A). The cytotoxicity of parasites grown with 1.6 mM Zn^{2+} was also reduced by 25%; however, only 50 µg/ml of antibodies was required to abolish this effect and similarly reduced cytotoxic effects were observed upon treatment with 75 µg/ml of antibody (Fig. 6B). In contrast, purified normal mouse sera (NMS) had no cytotoxic effect on parasite activity toward DU145 monolayers. To determine whether secreted native TvMP50 (Fig. 3) had a similar effect as endogenous (Fig. 6) and recombinant protein (Fig. 5A) toward DU145 cells, 400 µg of 6-hour secretions of parasites grown with or without Zn^{2+} were treated with 75 µg/ml of purified α-rTvMP50 or NMS antibodies and then incubated with DU145 monolayers in interaction media. The control NMS had no effect on secretions from conditions with or without Zn^{2+} conditions after 6 h of incubation (Fig. 6C circle 3, and D circle 6). In contrast, cells treated with secretions from without Zn^{2+} conditions that had been treated with 75 µg/ml of α-rTvMP50 antibody had significantly reduced visual damage (Fig. 6C, circle 2) compared with the negative control NMS treatment (Fig. 6C, circle 3), which had monolayer integrity similar to the untreated control DU145 cells (Fig. 6C, circle 1). More visual damage was observed for the DU145 monolayer treated with secretions from parasites grown with 1.6 mM Zn^{2+} and treated with NMS (Fig. 6D circle 6) than for the monolayer treated with secretions of parasites grown without Zn^{2+} (Fig. 6C, circle 3); however, a similar reduction in damage was observed upon the addition of 75 µg/ml of purified α-rTvMP50 antibody (Fig. 6D, circle 5). On the other hand, the secretions of female isolate parasites CNCD147 had less cytotoxic effects toward DU145 cells, even in $+Zn^{2+}$ conditions at 3 and 6 h of incubation treated with NMS (Fig. 7A, circle 2 and B, circle 5) in comparison with HGMN01 isolate (Fig. 6D), however, this cytotoxic effect was completely abolished with 50 µg/ml (Fig. 7A, circle 3) and 75 µg/ml of purified α-rTvMP50 antibody (Fig. 7B, circle 6) These results confirm our quantitative analysis of the antibody treatments, showing not only that the parasite interaction was responsible of cytotoxicity, but that TvMP50 must also be present in the secretions for this pathogenic mechanism.

3.6. *Endogenous T. vaginalis TvMP50 requires Zn^{2+} to exert cytotoxic activity toward DU145 cells*

Finally, to demonstrate the participation of Zn^{2+} in the TvMP50 interaction with and damage to DU145 cells, we analyzed the cytotoxicity of pre-treated parasites toward DU145 cells. Parasites that had been pre-treated with 5 mM EDTA destroyed 85% of the monolayer (Fig. 8, EDTA, grey bar), and EGTA pre-treated parasites showed decreased cytotoxicity, disrupting 70% of the monolayer (Fig. 8, EGTA, grey bar). Although these results show discrete inhibitions of *T. vaginalis* cytotoxicity toward DU145 cells, the treatment with the specific

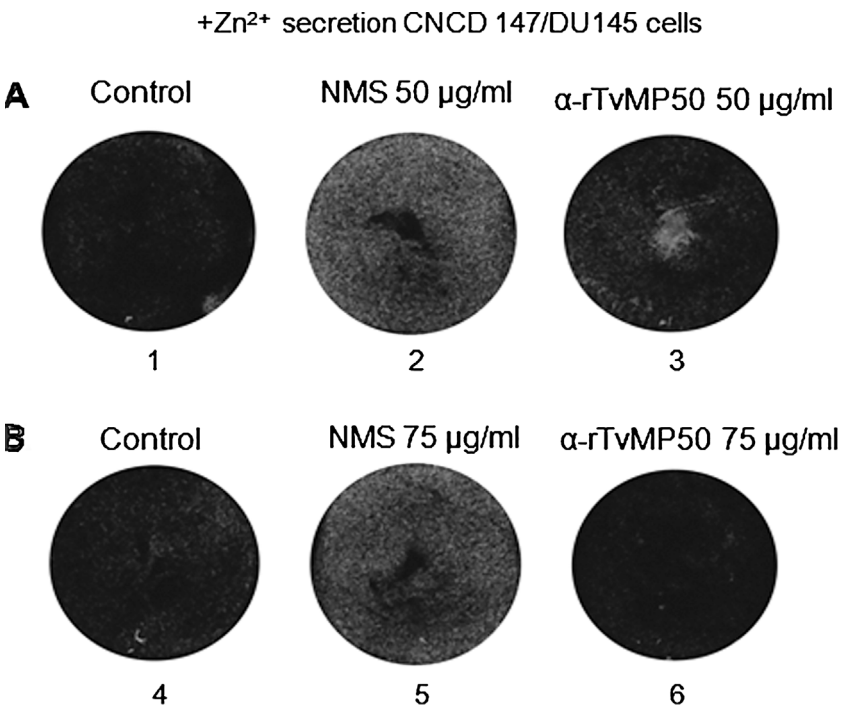

Fig. 7. Cytotoxicity of *T. vaginalis* secretions of CNCD 147 isolate toward DU145 cells. (A) A representative image of the visual cytotoxicity of 400 µg/ml of secretions of parasites with zinc ($+Zn^{2+}$) treated with 50 µg of α-rTvMP50 (circle 3) or with NMS (circle 2) co-incubated with DU145 cells 3 h. The non-treated DU145 cells monolayer fixed and crystal violet stained was used as monolayer Integrity control (circle 1). (B) Representative image of visual cytotoxicity of 400 µg/ml of secretions of parasites ($+Zn^{2+}$) treated with 75 µg of α-rTvMP50 (circle 6) or with NMS (circle 2) co-incubated with DU145 cells 6 h. Non-treated DU145 cells monolayer fixed and crystal violet stained was used as monolayer integrity control (circle 4).

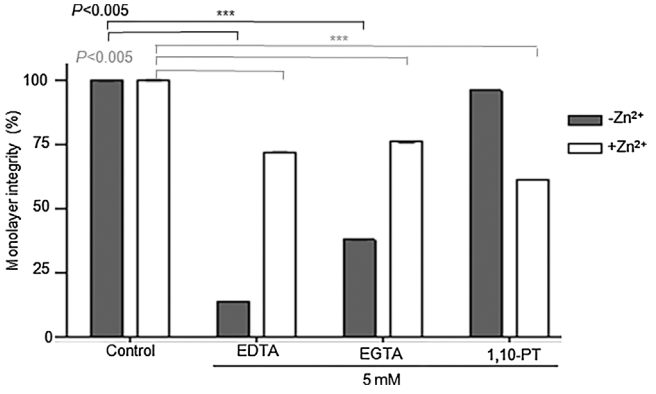

Fig. 8. The cytotoxic effect of TvMP50 of *T. vaginalis* toward DU145 cells requires Zn^{2+} presence. The quantification of cytotoxic effect of live parasites without zinc ($-Zn^{2+}$) (grey bars) and with zinc ($+Zn^{2+}$) (white bars) co-incubated 6 h with DU145 monolayer and treated EDTA, EGTA and 1,10-PT showing the reduced cytotoxic effect. The non-treated DU145 cells monolayer was used as control. Two independent assays in triplicate were performed and the error bars indicate the SD. A significant difference in cytotoxicity ($P < 0.005$) was found for the treatment with EDTA and EGTA without zinc ($-Zn^{2+}$) or treated with EDTA, EGTA and 1,10-PT treatment with zinc ($+Zn^{2+}$) marked with three asterisks compared with the control condition.

chelating agent 1,10-PT significantly protected the cell monolayer from *T. vaginalis* cytotoxicity, as only 10% of the monolayer was destroyed (Fig. 8, 1,10-PT, grey bar). This result indicates that the chelating agents negatively affect this *T. vaginalis* pathogenic mechanism by removing Zn^{2+} that could participate in *in vivo* TvMP50 metalloproteinase activity. This effect was verified using parasites grown in 1.6 mM Zn^{2+} conditions and using Zn^{2+} in the interaction media. Under these conditions, cytotoxicity was 25% even with EDTA and EGTA treatment, and cytotoxicity was 20% with 1,10-PT treatment (Fig. 7, black bars). These results demonstrate the participation of Zn^{2+} in *T. vaginalis* pathogenesis and that TvMP50 is a metalloproteinase that is expressed in the parasite and is considered a new parasite virulence factor.

4. Discussion

T. vaginalis is the causal agent of trichomoniasis and can damage host cells through several pathogenic mechanisms, such as cytotoxicity resulting in cervical epithelial cell death [42,43]. This event is primarily mediated by parasite CPs [18,44] and secretion/excretion products [45] including extracellular vesicles (EVs) and exosomes [46]. Endogenous and secreted parasite CPs have been established as virulence factors that are modulated by urogenital microenvironment conditions such as the presence of iron [47]. The *T. vaginalis* genome has been described as containing 446 genes that encode proteinases for which members of 13 MPs families have been identified; however, only the GP63-like proteinase has been characterized. The GP63-like proteinase induces apoptosis through the Mcl-1/Bim and Bcl-xl/Bim complexes [30] and disrupts the mTOR/p7056K pathway in cervical SiHa cells through mTOR cleavage [48]. Consequently, the secretions effect of *T. vaginalis* against host cervical cells has been described to include a parasite secretion stimulus with a possible immunomodulatory effect on the host cell secretion of proinflammatory cytokines interleukin-6 and −8 [46]. In contrast, the effects of other microenvironment stimuli, *e.g.*, Zn^{2+} and male urogenital tract cells, in the secretion of virulence factors such as metalloproteinases have not been studied. This work aimed to characterize the *T. vaginalis* 50 kDa metalloproteinase TvMP50 as a new endogenous and secreted virulence factor implicated in prostatic cell damage.

The *mp50* 5′-UTR analysis showed a possible Inr sequence (TCAYTTT) and three possible regulatory motifs [40]. Two M2 motifs are localized 14 and 41 nt upstream of the ATG initiation codon, respectively, and one M1 motif (CAAAATTT) localized 4 nt upstream of ATG site that might be the possible functional promoter of *mp50*. Woehle et al. proposed that M1 sequences in *T. vaginalis* might be used for CDS while the second M2 (41 nt upstream of ATG) sequence might be used to transcribe long non-coding RNA according to the frequency in RNAseq [49]. These data did not show a potential transcriptional start site (TSS). Huang et al. showed that *mp50* (TVAG_403460) was upregulated by glucose presence, and downregulated in glucose restriction conditions while the eleven *mp50* paralogues had different expression profiles, *e.g.* the metalloproteinase with access number TVAG_040850 had low expression in glucose rich conditions, but its expression was slightly higher in glucose restricted conditions with respect to *mp50* [50]. The Zn^{2+}-mediated upregulation of *mp50* is interesting, as it supports the hypothesis that *T. vaginalis* possesses Zn^{2+}-

responsive gene expression regulatory machinery that is at least similar to the mammalian transcriptional factor MTF-1 and a metal-responsive element in its 5′-UTR [51]. This mechanism is similar to the iron-mediated regulation of *ap65-1* adhesin [52] but is instead mediated by Zn^{2+} (work in progress). Furthermore, the TvMP50 protein was found to be overexpressed in the male isolate of *T. vaginalis*, showing a correlation between the gene expression and protein levels that might indicate transcriptional regulation and a potential mRNA stabilizing mechanism mediated by Zn^{2+}. Additionally, TvMP50 was localized to vacuole/vesicle-like structures where the TvMP50 signal was strong in the parasite cytoplasm. The presence of vacuolar structures could indicate Zn^{2+} accumulation in these structures. Until now, there have been no reports of zinc or other cation deposits in *T. vaginalis*; instead, acidocalcisomes in the bloodstream trypomastigotes of the protozoan pathogen *T. cruzi* were found to contain several cations, including calcium and zinc [53]. Although *T. vaginalis* also possesses lysosomes, organelles can act as zinc reservoirs in mammalian cells [54,55]; therefore, we cannot ignore the presence of TvMP50 in lysosomes as observed for *T. vaginalis* CPs [56–58]. TvMP50, based on Uniprot annotation (http://www.uniprot.org/uniprot/A2F8Y2), does not possess a signal peptide sequence that could indicate a possible canonical secretion pathway. However, their localization in vacuolar structures could suggest a secretion pathway mediated by exosomes and micro vesicles referred to as extracellular vesicles (EV) [59]. Previously, GP63-like and other metalloproteinase clans (MA and MH) were identified in EV [46], and TvMP50 could be also secreted by EV. The recent discovery of whole lysosome secretion during Zn^{2+} stress [58] is another potential route for proteinase secretion that prevents cation toxicity toward the parasite and results in indirect secretion of proteinases present in this organelle as virulence factors. Using our data, we concluded that TvMP50 functions during *T. vaginalis* infection to mediate proteolytic activity and cause host cell damage. As a result, and due to its previous identification in the immunoproteomes of male patients with trichomoniasis, we analyzed the possible secretion of this metalloproteinase from the parasite to the media [31] and tested the effects of Zn^{2+} on this secretion. The parasites secrete TvMP50 during short incubations, suggesting that TvMP50 secretion is not time-dependent unlike that observed for female isolate parasite CPs such as TvCP4, which are secreted in a time-dependent manner [56]. Gelatin-zymograms were performed to determine whether secreted TvMP50 was proteolytically active, and this analysis showed that this metalloproteinase is active both in the parasite and in the secretions. Evidence for this conclusion includes activity in the expected molecular weight region (50 kDa) and the effects of treatment with metallic chelants EDTA and EGTA. The use of these chelants in other studies of *T. vaginalis* and *Tritrichomonas foetus* facilitated the identification of two high molecular weight (142 and > 220 kDa) proteins as metalloproteins by inhibiting their proteolytic activity in zymograms [60]. Furthermore, a 1,10-PT treatment of parasites and secretions from normal (without Zn^{2+}) conditions completely abolished TvMP50 proteolytic activity compared with parasites and secretions from Zn^{2+} conditions. In contrast, proteolytic activity was unaffected in with Zn^{2+} conditions, and a new high molecular weight band of activity was present, suggesting that Zn^{2+} could also be activating other proteinases or affecting another zinc-dependent proteolytic activity. These data suggest two possibilities: first, 1,10-PT chelates the available Zn^{2+} in the parasite growth media and secretions in this condition [61]; second, at the concentration used, 1,10-PT also presents affinity for an active GP63-like metalloproteinase in the parasite [30], or even that other uncharacterized metalloproteinases allow the observation of persistent TvMP50 proteolytic activity.

Once we confirmed the TvMP50 presence in *T. vaginalis* secretions using proteolytic activity, we evaluated the effects of endogenous, secreted and recombinant TvMP50 on prostatic cells. rTvMP50 was observed to have a cytotoxic effect on DU145 monolayers after 6 h of incubation and the parasite secretions from HGMN01 and CNCD147 isolates grown with Zn^{2+} were also cytotoxic toward DU145 monolayers at 6 h. However, the secretions of the CNCD147 isolate were less cytotoxic. The cytotoxicity of the secretions parasite toward the DU145 cells was reduced by the α-rTvMP50 in both isolates, suggesting the participation of TvMP50 as a virulence factor. These data are consistent with our previous results in which *mp50*, the TvMP50 protein expression, and the cytotoxic effect were lower in the CNCD 147 isolate grown in Zn^{2+} presence [27]. The different cytotoxic effects between these two isolates might be by the modulation in the expression, protein levels, surface localization, secretion mechanism, and proteolytic activity of other proteinases such as CPs, TvCP65, and TvCP39 in Zn^{2+} presence and mediated by specific cell contact (cervical or prostatic) in addition to TvMP50 (work in progress).

These results suggest that similarly to the effects of GP63-like metalloproteinases on SiHa cells, the cytotoxic activity of TvMP50 toward DU145 cells results from the presence of metalloproteinase(s), possibly triggering cell death. The secretions of *T. vaginalis* contain multivesicular bodies, extracellular vesicles, factors such as BspA and cyclophilin, and proteinases, including the GP63-like MP, and several effects of these factors toward the parasites and host cells have been described (reviewed by [57]), indicating that the cytotoxic effect of secretions is a multifactorial process. Similarly, pretreatment of live parasites with chelants also negatively affected this pathogenic property, and a comparable effect was observed in the zymograms. These results show a difference from the GP63-like MP, for which treatment of live parasites with 1,10-PT did not induce SiHa cell death by mTOR cleavage [28]. TvMP50 activity was required to induce cellular detachment and death therefore, the precise mechanism of TvMP50-induced cellular damage must be analyzed and compared with the characterized proteins of the *T. vaginalis* secretome, and their relationships with the male urogenital tract where parasites can affect other important cells, *e.g.*, spermatozoids, leading to decreased sperm motility, viability and integrity, as well as decreased fertilization rates in mice at least *in vitro* [62]. This data suggests the importance of TvMP50 metalloproteinase during the *T. vaginalis* pathogenesis in Zn^{2+}. For this reason we suggest that TvMP50 could be included in the family of cytolytic effectors involved in the *T. vaginalis* pathogenicity mediated by environmental conditions such as the functional porins released by low pH [63], the 250 kDa cell free *T. vaginalis* culture factor TVF that induces rounding and clumping without cell lysis of target cells [64], the 200 kDa-glycoprotein cell-detaching factor (CDF) released into the medium by *T. vaginalis*, in contact with epithelial cells [65], the secreted macrophage migration inhibitory factor (TvMIF) with possible implications in extracellular matrix degradation and prostatic cancer [66]. and the EVs involve in several functions in parasitic diseases [67].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2017.09.001.

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