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Original article

The iron-induced cysteine proteinase TvCP4 plays a key role in *Trichomonas vaginalis* haemolysis

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Abstract

Trichomonas vaginalis has multiple proteinases, mainly of the cysteine type (CPs), including a 34 kDa precursor cathepsin L-like CP dubbed TvCP4. TvCP4 is an iron-up-regulated CP. The goal of this work was to identify the role of TvCP4 in the virulence of *T. vaginalis*. We cloned, expressed, and purified the recombinant mature enzyme region of TvCP4 (TvCP4r) to produce a rabbit polyclonal antibody (α-TvCP4r). This antibody reacted with a ~24 kDa protein band in total protein extracts that could correspond to the mature enzyme. By two-dimensional western blot assays TvCP4 corresponded to three protein spots of ~24 kDa with p*I* values of ~6.7, 6.9, and 7.0 and two spots of ~22 and ~21 kDa with a p*I* of 6.9, as confirmed by mass spectrometry. As expected, a higher amount of TvCP4 was detected in cytoplasmic vesicles, lysosomes, and on the surface of iron-rich parasites when compared with normal and iron-depleted parasites. The α-TvCP4r antibody protected human erythrocytes from trichomonal lysis. Additionally, TvCP4 is expressed during infection and is part of the released products detected in vaginal fluids of patients with trichomonosis. Thus, data show that TvCP4 is an iron-induced CP that participates in *T. vaginalis* haemolysis.

Keywords: *Trichomonas vaginalis*; Cysteine proteinases; TvCP4; Iron-induced CP; Haemolysis

1. Introduction

Trichomonas vaginalis is a flagellated protozoan parasite found in the human urogenital tract that is responsible for human trichomonosis, which is one of the most common, non-viral sexually transmitted infections (STI) worldwide. This infection is associated with severe health complications such as increased susceptibility to human immunodeficiency virus, infertility, and cervical and prostate cancer [1].

T. vaginalis has multiple proteinases, mainly of the cysteine type (CPs). In the *T. vaginalis* genome sequence, 48 genes encoding papain-like CPs were found [2]. Up to 23 spots with proteolytic activity between 23 and 110 kDa and p*I* values between 4.5 and 7.0 are detected using two-dimensional (2-D) substrate gel electrophoresis (zymograms) [3]. However, most of these spots are encoded by only nine distinct CP genes [4]. Some of these genes are differentially modulated by iron [5,6] and play crucial roles in characteristics related to the virulence of *T. vaginalis*, including cytoadherence [7–10], cytotoxicity [8,11–16], haemolysis [17–20], complement resistance [21], immune evasion [15,22] and apoptosis in human cells [23,24]. Additionally, trichomonad CPs are found in the vaginal secretions of patients with trichomonosis, some of which are immunogenic [4,10,11,15,16,25,26].

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For trichomonads, erythrocytes are an essential source of nutrients, such as lipids and iron. This parasite phagocytise and lyse erythrocytes by a contact-dependent mechanism [17,27–29]. Haemolysis requires calcium and an acidic pH (≤6.5) at 37 °C. Among the molecules possibly involved in *T. vaginalis* haemolysis, pore-forming proteins and phospholipase-A-like proteins have been identified as cytolytic factors [30,31]. Additionally, a triacylglycerol lipase (TvLIP) up-regulated by iron may also be a haemolytic factor in *T. vaginalis* [32]. Moreover, CPs participate in haemolysis, potentially through a contact-dependent mechanism [17]. A non-secreted 30 kDa CP that degrades spectrin is considered to be the main effector responsible for the cytoskeletal disruption associated with the cytolytic mechanisms of *T. vaginalis* [30]. Spectrin degradation occurs prior to the lysis of erythrocytes.

In addition, TvCP4 is a papain-like CP that is up-regulated by iron at the posttranscriptional level through an IRE/IRP-like system similar to that described for iron homoeostasis in mammalian cells [33]. Recently, TvCP4 has been reported as one of the most immunogenic trichomonad CPs recognised by trichomonosis patient sera [4]. However, the function of TvCP4 in the virulence of *T. vaginalis* has not yet been determined.

In this work, we showed that TvCP4 is in cytoplasmic vesicles, lysosomes and at the parasite surface, released in vitro, present in vaginal secretions, and is a virulence factor playing a role in haemolysis, under iron-rich conditions.

2. Materials and methods

2.1. Parasite culture

T. vaginalis parasites from the fresh clinical isolate CNCD 147 were used in this study for all experiments [11]. Trichomonad cultures were maintained for up to two weeks by daily passage in trypticase–yeast extract–maltose (TYM) medium supplemented with 10% heat-inactivated horse serum (HIHS) at 37 °C [34]. Only organisms at the mid-logarithmic phase were used for all assays. The TYM–HIHS medium contained 20 μM iron (normal growth condition) [12,35]. For parasites that were grown under iron-rich or iron-depleted conditions (250 or 0 μM iron, respectively), the culture medium was supplemented with 250 μM ferrous ammonium sulphate or 100 μM 2,2′-dipyridyl (Sigma–Aldrich, Co., St. Louis, MO, USA), respectively, 24 h prior to inoculation with parasites [12].

2.2. Cloning and expression of the mature TvCP4 recombinant proteinase

A fragment (657 bp) of the *tvcp4* gene (GenBank accession number AY679763) [33] encoding the mature region of TvCP4 (aa 87–305) was amplified by PCR using trichomonad genomic DNA as a template and sense primer 5′-GAGCTTGGATCCATCGCTAACGCTGACTGCGAC-3′ and antisense primer 5′-GAGCTTAAGCTTTTACTTGTCCTGT GGGATGCA-3′. The restriction sites used were *Bam*HI (sense) and *Hind*III (antisense), which are underlined in the primer sequences. The amplicon encoding the TvCP4 mature enzyme was directionally cloned into the pCold I prokaryotic expression vector (Takara Bio Inc., Mountain View, CA, USA), as recommended by the manufacturer. Prior to bacterial expression, candidate clones were sequenced using an Applied Biosystems AB1377 Automatic Sequencer (UNAM, Institute of Cell Physiology) to select the clones containing the correct insert. Recombinant protein expression in the *Escherichia coli* strain BL21 (DE3) was induced by an addition of 1 mM IPTG for 16 h at 37 °C and analysed by SDS–PAGE and western blot (WB) assays. The WB assays were performed by using an anti-histidine monoclonal antibody (α-His) as the primary antibody and a peroxidase-conjugated goat anti-mouse polyclonal secondary antibody (Invitrogen-Gibco, Carlsbad, CA, USA) at a dilution of 1:3000. The mature TvCP4 recombinant protein (TvCP4r) was purified by affinity chromatography using Ni-Sepharose 6 Fast Flow columns (GE Healthcare), according to the manufacturer's instructions.

2.3. Production of a polyclonal anti-TvCP4r antibody (α-TvCP4r)

For α-TvCP4r antibody production, a four-week-old male New Zealand rabbit was subcutaneously immunised seven times at two-week intervals using 0.15 mg of purified TvCP4r protein homogenised with Freund's complete or incomplete adjuvant (Gibco) at a ratio of 1:1 [36]. This anti-serum (α-TvCP4r) was used in WB, indirect immunofluorescence, and haemolysis assays. Pre-immune (PI) rabbit serum obtained prior to the immunisation schedule was used as a negative control in all experiments with rabbit antibodies. For the haemolysis assays, IgG fractions were obtained by using the caprylic acid method [36]. The use and handle of rabbits for antibody production was included in a protocol submitted and approved by the Institutional Animal Care and Use Committee dubbed "CICUAL" at CINVESTAV.

2.4. Protein preparation and 1-D and 2-D gel electrophoresis

Total protein extracts (TPE) were obtained from 2×10^7 parasites grown in different iron concentrations using 10% trichloroacetic acid (TCA) precipitation at 4 °C overnight, and analysed using 1-D SDS–PAGE on 12% polyacrylamide gels. The proteolytic activity of trichomonad supernatants previously lyophilised after the in vitro secretion assays was analysed using 1-D substrate SDS–PAGE on 12% polyacrylamide gels copolymerised with 0.2% gelatine (Bio-Rad Laboratories, Hercules, CA, USA). Proteinases were renatured with 2.5% Triton X-100 and activated with 100 mM sodium acetate buffer, pH 4.5, containing 0.1% β-mercaptoethanol for 18 h at 37 °C as previously described [11]. Zymograms stained with Coomassie brilliant blue (CBB) showed proteolytic activity as white bands against a dark background. The experiments were performed at least three times with similar results.