International Journal of Biochemistry and Cell Biology 102 (2018) 87–100

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Characterization of a novel endogenous cysteine proteinase inhibitor, trichocystatin-3 (TC-3), localized on the surface of *Trichomonas vaginalis*

Diana Belén Sánchez-Rodríguez[a], Jaime Ortega-López[b], Rosa Elena Cárdenas-Guerra[a], Gerardo Reséndiz-Cardiel[b], Bibiana Chávez-Munguía[a], Anel Lagunes-Guillen[a], Rossana Arroyo[a,*]

[a] *Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Av. IPN 2508, Col. San Pedro Zacatenco, Delg. Gustavo A Madero, CP 07360, Mexico City, Mexico*

[b] *Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Av. IPN 2508, Col. San Pedro Zacatenco, Delg. Gustavo A Madero, CP 07360, Mexico City, Mexico*

ARTICLE INFO

Keywords:
Cystatin-like CP endogenous inhibitor
Cytotoxicity
Golgi complex
Surface protein
Trichocystatin-3
Trichomonas vaginalis

ABSTRACT

Trichomonas vaginalis is a flagellated protist responsible for human trichomoniasis. *T. vaginalis* has three genes encoding for endogenous cysteine proteinase (CP) inhibitors, known as trichocystatin-1 through trichocystatin-3 (TC-1, TC-2, and TC-3). These inhibitors belong to the cystatin family. In this study, we characterized trichocystatin-3 (TC-3), an endogenous cysteine proteinase (CP) inhibitor of *T. vaginalis*. TC-3 possesses a signal peptide in the N-terminus and two putative glycosylation sites (typical of family 2, cystatins) but lacks the PW motif and cysteine residues (typical of family 1, stefins). Native TC-3 was recognized as an ~18 kDa protein band in a *T. vaginalis* protein extract. By confocal microscopy, endogenous TC-3 was found in the Golgi complex, cytoplasm, large vesicles, and the plasma membrane. These localizations are consistent with an *in silico* prediction. In addition, the purified recombinant protein (TC-3r) functions as an inhibitor of cathepsin L CPs, such as human liver cathepsin L and trichomonad CPs, present in a proteinase-resistant extract (PRE). *Via* a pull-down assay using TC-3r as bait and PRE, we identified several trichomonad CPs targeted by TC-3, primarily TvCP3. These CP-TC-3 interactions occur in vesicles, in the cytoplasm, and on the parasite surface. In addition, TC-3r showed a protective effect on HeLa cell monolayers by inhibiting trichomonad surface CPs involved in cellular damage. Our results show that the endogenous inhibitor TC-3 plays a key role in the regulation of endogenous CP proteolytic activity.

1. Introduction

Trichomonas vaginalis is a flagellated protist responsible for human trichomoniasis, the most common non-viral sexually transmitted infection (STI) worldwide (WHO, 2012). This parasite infects the genitourinary tract of females and males. Over 440 protease genes have been found in the *T. vaginalis* genome (Carlton et al., 2007), and more than half of these proteases are cysteine proteinase (CPs). CPs play an essential role in the pathogenesis of this parasite, participating in processes such as cytoadherence (Arroyo and Alderete, 1989, 1995; Mendoza-López et al., 2000; Hernandez et al., 2004; Rendón-Gandarilla et al., 2013), hemolysis (Dailey et al., 1990; Fiori et al., 1993, 1996; Cárdenas-Guerra et al., 2013), cytotoxicity (Arroyo and Alderete, 1995; Alvarez-Sánchez et al., 2000, 2007; Hernández-Gutiérrez et al., 2003, 2004; Ramón-Luing et al., 2011), and apoptosis (Sommer et al., 2005; Kummer et al., 2008), among others.

Although the proteolytic activity of CPs is essential for trichomonal virulence, this activity must be strictly regulated to protect and maintain parasite homeostasis. *T. vaginalis* has three genes encoding endogenous CP inhibitors, known as trichocystatin-1 through trichocystatin-3 (TC-1, TC-2, and TC-3) (Carlton et al., 2007; Puente-Rivera et al., 2014). These inhibitors belong to the cystatin superfamily of endogenous CP inhibitors, which is widely distributed in a variety of organisms. These inhibitors are divided into three families. Stefins

Abbreviations: α-TC-3r, anti-TC-3r polyclonal antibody; CPE, clarified protein extract; CPs, cysteine proteinases; LC-ESI-MS/MS, liquid chromatography-Electrospray ionization-tandem mass spectrometry; ER, endoplasmic reticulum; G, Golgi complex; H, hydrogenosome; IFA, immunofluorescence assay; N, nucleus; PM, plasma membrane; PI, preimmune; PRE, protease-resistant extract; Rα-TvCP3r, rabbit anti-TvCP3 polyclonal antibody; SP, signal peptide; TC-3r, trichocystatin-3 recombinant protein; TEM, transmission electron microscopy; TvCP3, *T. vaginalis* CP3; TvCP4, *T. vaginalis* CP4; TvCP39, *T. vaginalis* CP39; TvLEGU-1, *T. vaginalis* legumain-1; V, vesicles

* Corresponding author.
E-mail address: rarroyo@cinvestav.mx (R. Arroyo).

https://doi.org/10.1016/j.biocel.2018.07.005
Received 18 February 2018; Received in revised form 12 July 2018; Accepted 13 July 2018

(family 1) are low molecular weight proteins that lack disulfide bonds, are non-glycosylated, and are mainly intracellularly located. Cystatins (family 2) are low molecular weight proteins with an N-terminal signal peptide (SP) and disulfide bonds, are usually non-glycosylated, and are extracellularly located or secreted. Some cystatins may form oligomeric structures by a domain swapping mechanism. Kininogens (family 3) are high and low molecular weight proteins with multidomains and glycosylation sites (Ekiel et al., 1997; Abrahamson et al., 2003; Sanders et al., 2004; Oliveira et al., 2003; Turk et al., 2008).

We previously reported the identification and characterization of trichocystatin-2 (TC-2). TC-2 was classified in family 1 as a stefin-like inhibitor. TC-2 showed inhibitory activity against papain, human cathepsin-L, and trichomonad cathepsin-L-like CPs. Additionally, it was shown that TvCP39, a cytotoxic protease (Hernández-Gutiérrez et al., 2004; Ramón-Luing et al., 2011), is one of the proteases targeted by TC-2. Parasite pretreatment with recombinant TC-2 inhibited *T. vaginalis* cytotoxicity (Puente-Rivera et al., 2014).

Here, we report the characterization of a novel endogenous inhibitor of surface CPs, trichocystatin-3 (TC-3). Its amino acid sequence is more divergent than those of the other two trichocystatins. TC-3 has a signal peptide at the N-terminus and two putative glycosylation sites, but not disulfide bonds, as its amino acid sequence lacks cysteine residues. It showed inhibitory activity against cathepsin-L-like proteases and it was localized in the cytoplasm and on the surface of *T. vaginalis*.

2. Materials and methods

2.1. Parasites and HeLa culture

The fresh clinical *T. vaginalis* isolate CNCD 280 (a type 1 isolate) (Conrad et al., 2012) was used in this study. It was maintained in *in vitro* culture for up to two weeks at 37 °C in trypticase-yeast extract-maltose (TYM) medium supplemented with 10% heat-inactivated adult bovine serum (HIABS) (Diamond, 1957). Mid-logarithmic phase parasites were used for all assays. HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Lab., Grand Island, NY) supplemented with 10% HIABS at 37 °C in a 5% CO_2 atmosphere (Arroyo et al., 1992; Alvarez-Sánchez et al., 2000).

2.2. Sequencing, phylogenetic analysis, structure prediction, and molecular cloning

The multiple alignment and evolutionary analysis of TC-3 were done using amino acid sequences of cystatins from several organisms, including the three trichocystatin sequences: TC-1 (TVAG_127040), TC-2 (TVAG_272260) (Puente-Rivera et al., 2014), and TC-3 (TVAG_034880). The phylogenetic tree was generated using the neighbor-joining method with the Molecular Evolutionary Genetic Analysis 7 (MEGA 7) program (http://www.megasoftware.net/). Bootstrap proportions were used to assess the robustness of the tree with 1000 bootstrap replications. Analysis of the 5′ and 3′ regulatory regions of the *tvicp-3* gene was performed with the reported sequence in the *T. vaginalis* database with the accession number TVAG_034880 (Carlton et al., 2007; www.Trichdb). The deduced amino acid sequence of TC-3 was used to predict post-translational modifications using SignalP and YinOYang 1.2 software. To predict the three dimensional (3D) structure model of native TC-3, Stefin B, and cystatin C, we use I-TASSER (Iterative Threading ASSEmbly Refinement; https://zhanglab.ccmb.med.umich.edu/I-TASSER/) server (Zhang, 2008). The models were visualized with PyMOL (TM), Version 1.3 (Schrödinger, LLC, USA).

The 315-bp gene encoding TC-3 was amplified from CNCD 280 *T. vaginalis* genomic DNA by PCR using specific primers (sense: 5′-GGCG GGATCCATGATTCCACTCTTGATGCTCTTC-3′; and antisense: 5′-GGCG TCTAGATCAAGCTTTGATC-3′) that included restriction sites for *Bam*HI and *Xba*I respectively. We used the following cycling conditions: 2.5 min at 94 °C; 32 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and a final cycle for 7 min at 72 °C. The full-length 957-bp gene encoding TvCP3 (TVAG_090100) was amplified by PCR from CNCD 147 *T. vaginalis* genomic DNA using specific primers (sense: 5′-GAGCTTGG ATCCATGTTCTCTGCATTCTTTGCT-3′; antisense: 5′-GAGCTTCTGCAG TTACTCGACCTGTGGGATGAT-3′) that included restriction sites for *Bam*HI and *Pst*I, respectively. We used the following cycling conditions: 2 min at 95 °C; 32 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C; and a final cycle for 7 min at 72 °C. Both amplicons were cloned into the pGEM-T Easy vector (Promega, A1360) and transformed into *Escherichia coli* DH5α competent cells. Positive clones for each gene were selected, and the inserts were sequenced in an AB1377 Applied Biosystems Automatic Sequencer (UNAM, Instituto de Biología, Mexico). The multiple alignment and insert sequence analyses were performed using DNAMAN Version 3.0 (Lynnon Biosoft) software.

2.3. RT-PCR assays

Total RNA was extracted from CNCD 280 (type 1) and CNCD 147 (type 2) parasite isolates using TRIzol (Sigma). For cDNA synthesis, we used SuperScript® II Reverse Transcriptase (Invitrogen Technology, Carlsbad, CA, USA), following the manufacturer's instructions. A total of 500 ng of cDNA was used as a template to amplify the *tvicp-3* gene by PCR using specific primers with the same cycling conditions described above for PCR. *T. vaginalis* β-tubulin mRNA (Madico et al., 1998) was used as an internal control. Amplified cDNA products were separated on a 1.5% agarose gel with ethidium bromide.

2.4. Expression and purification of recombinant trichocystatin-3 (TC-3r) and recombinant TvCP3 proteins

The complete *tvicp-3 or tvcp3* inserts cloned into the pGEM-T Easy vector were digested with the appropriate restriction enzymes and subcloned into the pCold1 expression vector (Takara Bio Inc., Mountain View, CA, USA). The pCold1-*tvicp-3* or pCold1-*tvcp3* construct was transformed into *E. coli* BL21 (C41) or BL21 (DE3) competent cells, respectively. The expression of the recombinant TC-3 protein (TC-3r) was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 16 °C for 5 h, whereas the expression of the recombinant TvCP3 protein (TvCP3r) was induced with 1 mM IPTG at 37 °C for 22 h. The TC-3r protein in the soluble fraction and the TvCP3r protein from solubilized inclusion bodies were purified by affinity chromatography using Ni-Sepharose 6 Fast Flow columns (His-Trap FF; GE-Healthcare-Amersham Biosciences, UK), following the manufacturer's instructions. The purity of the eluted TC-3r and TvCP3r proteins was determined by SDS-PAGE separation on a 12% polyacrylamide gel, Coomassie brilliant blue (CBB) staining, and Western blot (WB) assays using an anti-His 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 1:3000 dilution.

2.5. Production of polyclonal antibodies against TC-3r (anti-TC-3r) and TvCP3r (anti-TvCP3r)

A two-month-old female New Zealand rabbit was subcutaneously immunized using 150 μg of purified TC-3r as an antigen emulsified with TiterMax Gold adjuvant (Sigma-Aldrich), following the manufacturer's instructions. The rabbit was bled every fifteen days for three months. For mouse anti-TC-3r antibody production, ten six-week-old Balb/c mice were subcutaneously immunized using 50 μg of TC-3r emulsified with TiterMax Gold adjuvant per animal. The mice were bled fifteen days after the last immunization. To generate a polyclonal antibody against TvCP3r (anti-TvCP3r), a six-week-old male New Zealand rabbit was intramuscularly immunized twice every fifteen days with 0.5 mg of purified TvCP3r plus TiterMax Gold adjuvant (Sigma-Aldrich). The anti-TvCP3r immune serum was obtained fifteen days after the last