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Experimental Parasitology 111 (2005) 143–153
Experimental Parasitology
www.elsevier.com/locate/yexpr

Theileria orientalis: Cloning a cDNA encoding a protein similar to thiol protease with haemoglobin-binding activity

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Received 11 February 2005; received in revised form 31 May 2005; accepted 7 June 2005
Available online 1 September 2005

Abstract

A gene encoding a protein (Tocp1) from *Theileria orientalis* was isolated from a cDNA library and the deduced amino acid sequence of Tocp1 has 476 amino acids. The primary structure of Tocp1 is similar to eukaryotic thiol proteases (EC 3.4.22.-), but no enzymatic activity was observed with the substitution of essential cysteine at the cysteine active site for glycine. Southern blot analysis showed that multiple genes similar to Tocp1 were present in the parasite genome. Sequence analysis of the genome of the parasite showed that there are at least five different genes similar to Tocp1. Tocp1 transcripts were detected in the *T. orientalis* piroplasma by Northern blot analysis. Western blot analysis showed that Tocp1 was expressed in the piroplasm of *T. orientalis*. To address the role of Tocp1 in the life cycle of *T. orientalis*, Tocp1 was expressed using pET32 expression system. Binding affinity to haemoglobin was demonstrated by enzyme-linked immunosorbent assay.

Index Descriptors and Abbreviations: *Theileria orientalis*; Cysteine proteinase; Haemoglobin-binding protein; Tocp1, *Theileria orientalis* cysteine proteinase-like protein 1 (GenBank Accession No. 15AB9780); rTocp1, recombinant Tocp1; tTocp1, truncated Tocp1; PCR, polymerase chain reaction; TRX, thioredoxin protein; HgbA, haemoglobin-binding protein A of *Pasteurella multocida*; KGP, lysine-gingpain of *Porphyromonas gingivalis*; HbR, haemoglobin receptor domain protein of *Porphyromonas gingivalis*; HP, bovine haptoglobin; SERA, serine repeat antigen of *Plasmodium falciparum*; Tacp, *Theileria annulata* cysteine protease (GenBank Accession No. M86659); Tpcp, *Theileria parva* cysteine protease (GenBank Accession No. M37791); p34, soybean oil body 34 kDa protein (GenBank Accession No. A37126); ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

1. Introduction

Theileria spp. are a tick-transmitted protozoan parasite of cattle. There are several species of *Theileria* reported in cattle (Onuma et al., 1998) including *Theileria annulata*, *Theileria parva*, and *Theileria orientalis*. The *T. annulata* and *T. parva* are virulent while *T. orientalis* (*T. sergenti*) is a conditional pathogenic parasite, though it has been reported to occasionally cause some economic losses in grazing cattle in Japan (Minami et al., 1980; Onuma et al., 1998). Calves infected with *T. orientalis* suffer from anemia with fluctuating parasitemia over time, and occasionally death.

During the intraerythrocytic phase, haemoglobin is the major cytosolic protein accessible for parasites. Haemoglobin can be broken down into heme and globin, and this process is thought to be a potential target for anti-protozoa drug design. Haemoglobin can be degraded and utilized in food vacuoles by *Plasmodium* spp. (Daniel et al., 1990; Olliaro and Goldberg, 1995; Rosenthal and Meshnick, 1996).

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0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.06.003

Leishmania spp. absorb haemoglobin by endocytosis mediated through a protein located in the flagellar pocket (Shantanu et al., 1999). Gram-negative bacteria can acquire iron-containing compounds by different strategies (Maria Silvia et al., 2001). (1) The direct binding of iron-containing protein compounds to outer membrane receptors, or (2) the extracellular secretion of heme-binding proteins called "hemophores" that subsequently transport and deliver heme to their cognate outer membrane receptor. An anaerobic Gram-negative bacterium, *Porphyromonas gingivalis*, uptakes and utilizes haemoglobin and heme by expressing a haemoglobin-binding protein (HmuR) and a cysteine proteinase (gingipain) (Olczak et al., 2001). Haemoglobin-binding activity of gingipain is also demonstrated by others (Kuboniwa et al., 1998). In Gram-positive bacteria, proteins and genes involved in heme or haemoglobin utilization have been described only in *Corynebacterium* species (Drazek et al., 2000; Schmitt, 1997) and *Streptococcus pneumoniae* (Brown et al., 2001; Tai et al., 1993, 1997). Surface protein, SiaA, could specifically bind haemoglobin for streptococcal iron acquisition (Christopher et al., 2003).

To address how *T. orientalis* accesses and utilizes haemoglobin as the major nutrient source in the red blood cells, a cDNA library of *T. orientalis* piroplasma was constructed, and a gene encoding cysteine proteinase-like protein was cloned and its binding activity to haemoglobin was observed in the present work.

2. Materials and methods

2.1. Parasites

Preparation and purification of *T. orientalis* was done as previously described (Sako et al., 1999). Briefly, a splenectomized calf was challenged with C type of *T. orientalis*. When the calf developed appreciable parasitemia, non-coagulanted blood was collected and white blood cells were removed by filtration, while the infected erythrocytes were lysed with liquid nitrogen at 1000 psi for 2 min. *T. orientalis* were then purified from the lysate by differential centrifugation.

2.2. Extraction of DNA from T. orientalis and bovine white blood cells

Isolation of DNA from *T. orientalis* and bovine white blood cells was done using the *SepaGene* kit (Sanko Junyaku, Japan) following the manufacturer's instructions.

2.3. Extraction of total RNA and preparation of cDNA library

Isolation of total RNA from *T. orientalis* was done using the TRIzol Reagent (Gibco-BRL) following the instructions of the manufacturer, and treating with DNase I (Sigma–Aldrich) at 37 °C for 30 min. The cDNA library of the parasite was made using the SMART cDNA library construction kit (Clontech Laboratories, USA) according to the instructions of the manufacturer. Briefly, 1 μg of total RNA was mixed with the primer (CDIII/3′ PCR Primer) and the SMART III Oligonucleotide, and first-strand synthesis was performed at 42 °C for 1 h. cDNA amplification by LD PCR (at 95 °C for 5 s for initial denaturation, then at 95 °C for 5 s, and at 68 °C for 6 min for 20 cycles) using the primers in the kit. The product was treated with protease K at 45 °C for 20 min, purified, and digested with *Sfi*I. The restriction enzyme digestion product was size-fractionated using CHROMA SPIN-400 (Clontech Laboratories). The fractionated cDNA fragments were ligated into pDNR-LIB donor vector at 16 °C overnight and finally the recombinant vectors were transfected into *Escherichia coli* DH5α.

2.4. DNA sequencing analysis

2.4.1. Random sequencing analysis of the colonies in the cDNA library

To investigate the expression profile of proteins in piroplasm of *T. orientalis*, random sequencing analysis of the colonies in the cDNA library was carried out. Using the Dye Terminator cycle sequencing system (Perkin Elmer-Applied Biosystems, USA) and automated sequencers (Perkin Elmer-Applied Biosystems 310 Genetic Analyzer), the nucleotide sequence of the cloned cDNA was determined using the vector-specific primers of SMART cDNA library construction kit (Clontech Laboratories, USA). The template for sequencing was generated by PCR amplification of the cloned DNA using the vector-specific primers (at 94 °C for 1 min as initial denaturation; then 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min during 25 cycles; then at 72 °C for 7 min). After purification, sequencing PCR was run using DNA sequencing kit (Applied Biosystems) at 96 °C for 15 s; 50 °C for 15 s; and 60 °C for 4 min repeated 25 times. Sequence analysis was done using the Genetic Mac software package version 10 (Software development, Japan) as well as the GenBank and the Swissprot databases for comparison of the cysteine protease predictive amino acid sequence with other known proteins in the database.

2.4.2. DNA sequencing analysis of Tocp1 in the genomic DNA of T. orientalis

To verify that the substitution of glycine for cysteine at the active site cysteine is not an artifact, according to the cDNA sequence of Tocp1, a series of primers (F1 5′-ATGGCTGATGCAGTTTTGTC-3′; F2 5′-CATCTTATTGGCTGTCACGGC-3′; R1 5′-CTTGGACTTGAAGTCGCAG-3′; R2 5′-GAAGGACTTCTTGCTGG