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Leishmania aethiopica: Identification and characterization of cathepsin L-like cysteine protease genes ☆

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Abstract

There is limited information on the biology and pathogenesis of *Leishmania aethiopica*, causative agent of cutaneous leishmaniasis (CL) in Ethiopia. In this study we have identified and characterized two cathepsin L-like cysteine protease genes, Lae*cpa* and Lae*cpb*, from *L. aethiopica*. The predicted amino acid sequence of Lae*cpa* and Lae*cpb* is more than 75% identical with homologous cathepsin L-like cysteine protease genes of other *Leishmania* species and less than 50% identical with human cathepsin L. Lae*cpa* is expressed predominantly in the stationary, and to a lower level, during the amastigote stage while Lae*cpb* is specifically expressed in the stationary stage of *L. aethiopica* development. Phylogenetic analysis showed that the two genes are grouped into separate clades which are the result of gene duplication. The isolation of these genes will be useful in developing *Leishmania* species specific diagnostics for molecular epidemiological studies and serves as a first step to study the role of cysteine proteases in *L. aethiopica* pathogenesis.

Index Descriptors and Abbreviations: *Leishmania aethiopica*; Cysteine Proteases; Cathepsin L; CL, Cutaneous leishmaniasis

1. Introduction

Leishmaniasis is a disease caused by flagellated protozoan parasites of the genus *Leishmania*, which belong to the order kinetoplastida and family trypanosomatidae. Leishmaniasis is prevalent in 88 countries of which 72 are developing countries (Desjeux, 1996; WHO, 1998). Both visceral and cutaneous forms of leishmaniasis are prevalent in Ethiopia. Visceral leishmaniasis is mainly caused by *Leishmania donovani* in Ethiopia (Ayele and Ali, 1984; Hailu et al., 1996). All the three forms of cutaneous leishmaniasis (localized, mucocutaneous and diffused) found in Ethiopia are mainly caused by *Leishmania aethiopica* and occasionally due to *Leishmania major* and *Leishmania tropica* (Sarojini et al., 1984). Fast and reliable species-specific tools are not available for diagnosis and identification of *L. aethiopica*. Moreover, cutaneous Leishmaniasis caused by *L. aethiopica* usually responds poorly to conventional doses of antimonial drugs and relapsing is common after treatment (Chulay et al., 1983). There is no effective vaccine for any form of leishmaniasis to date.

Cysteine proteases are one of the potential diagnostics, drug and vaccine candidates being studied extensively in different pathogenic organisms. They have been reported

☆ Nucleotide sequence data reported in this paper are available in the GenBank. The accession numbers are DQ071679 for Laecpa, DQ071678 for Laecpb, DQ071680 for Lmacpa and DQ286773 for Ltrcpb.

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from bacteria (Morihara, 1974), viruses (Bazan and Fletterick, 1988), fungi (Apodaca and McKerrow, 1989) and protozoan parasites (Omara-Opyene and Gedamu, 1997; Robertson and Coombs, 1994). Parasite cysteine proteases have been implicated in several processes including differentiation, nutrition, host cell infection, and evasion of the host immune response (Klemba and Goldberg, 2002; Mottram et al., 2004; Sajid and McKerrow, 2002). Cathepsin L-like cysteine proteases (CPA and CPB) have been shown to be necessary for the survival of *Leishmania mexicana* within macrophages *in vitro* (Denise et al., 2003). Knockout studies in *L. mexicana* have also shown that cathepsin L-like cysteine proteases act as modulators of host immune responses (Alexander et al., 1998; Buxbaum et al., 2003). Knockout and antisense mRNA inhibition studies of *Leishmania chagasi cpa* (LdcCys2) indicated that cysteine proteases help in the infection and survival of amastigotes within macrophages (Mundodi et al., 2005). Furthermore, the fact that cathepsin L-like cysteine proteases from different *Leishmania* species exhibit PCR-RFLP suggests their potential for the development of specific molecular diagnostics (Tintaya et al., 2004).

Leishmania CPA cysteine proteases are encoded by a single gene and characterized by having short C-terminal extension of only 10 amino acids. The expression of CPA is constitutive in *L. mexicana* while it is strictly amastigote specific in *L. chagasi* (Mottram et al., 1992; Mundodi et al., 2005). *Leishmania* CPB is the major cathepsin L-like cysteine protease having long C-terminal extensions and encoded by a multiple copy of 5 in *L. chagasi*, 6 in *L. donovani* (Mundodi et al., 2002), and 19 in *L. mexicana* (Souza et al., 1992). *L. donovani* and *L. chagasi* CPB expression is mainly promastigote stage while *L. mexicana* CPB expression is amastigote stage.

In the present study, we have identified and studied the expression and evolutionary classification of two distinct cathepsin L-like cysteine protease genes, Lae*cpa* and Lae*cpb* from *L. aethiopica*. This study will be useful in developing species specific molecular diagnostics and serves as a first step to study the role of cysteine proteases in *L. aethiopica* pathogenesis.

2. Material and methods

2.1. Cell culture

Leishmania aethiopica 1093/02 isolate, typed by isoenzyme electrophoresis (Genetu et al., 2006), was cultured at 26 °C in complete RPMI medium (which consists of RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 2 mM glutamine (Flow Laboratories), 100 U/ml penicillin (Gibco- BRL) and 100 μg/ml streptomycin (Gibco-BRL)). The Human monocyte cell line THP1 (ATCC), was used to generate the amastigote stage of *L. aethiopica*. THP1 cells were cultured in complete RPMI medium at 37 °C in the presence of 5% CO_2.

2.2. Infection of THP1 cells

Infection of THP1 cells was carried out as previously described (Mohamed et al., 1992). Briefly, THP1 cells were washed two times with RPMI-1640 at 1600 rpm in Allegra6R™ centrifuge (Beckman Coulter) for 10 min. The cells were resuspended at a concentration of 2×10^5 viable cells per ml with complete RPMI medium. Viability was determined using trypan blue. Retinoic acid (Sigma) was added to a final concentration of 1×10^{-6} M to differentiate the cells. The retinoic acid treated cells were incubated at 37 °C in 5% CO_2 for 5 days. The differentiated THP1 cells were washed three times at 1600 rpm in Allegra6R™ centrifuge (Beckman Coulter) for 10 min with RPMI-1640 supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were then resuspended in complete RPMI medium to a final concentration of 2×10^5 viable cells per ml and incubated for 30 min at 37 °C in 5% CO_2. Differentiation and viability of THP1 cells were checked using Wright stain and trypan blue exclusion respectively. Stationary phase promastigote *L. aethiopica* were pelleted at 3000 rpm for 20 min and resuspended in complete RPMI medium. The differentiated THP1 cells were incubated for 16 h at 37 °C in 5% CO_2 with live stationary *L. aethiopica* parasites at a parasite to cell ratio of 20:1. Free parasites were removed by washing three times with RPMI at 600 rpm and 4 °C for 10 min. Infection was monitored by cytospin preparation and Wright stain. Infected cells were cultured for 3 days and total RNA was extracted for RT-PCR analysis.

2.3. DNA and RNA extraction

Genomic DNA was isolated from early stationary phase parasite as previously described (Omara-Opyene and Gedamu, 1997). Briefly, about 10^{10} promastigotes were centrifuged at 3000 rpm in Allegra6R™ centrifuge (Beckman Coulter) for 20 min. The pellet was suspended in 1 ml lysis buffer (10 mM Tris–HCl (pH 8.3), 50 mM EDTA (pH 8.0), 1% SDS) and incubated with 1 mg of RNAse A (Sigma) at 37 °C for 1 h. Proteinase K (Roche Applied Bioscience) was then added to a final concentration of 100 μg/ml and incubated over night at 42 °C. Equal volumes of Phenol/chloroform/isoamylalcohol (25:24:1; Sigma) was added and centrifuged at 12,000*g* in a microcentrifuge (Eppendorf). The aqueous phase was transferred to a clean Eppendorf tube. The DNA was precipitated from the aqueous phase with isopropanol for 30 min at −20 °C, centrifuged at 12,000*g* at room temperature and the supernatant was discarded. The pellet was washed with 75% ethanol and centrifuged at 12,000*g* at room temperature and the supernatant discarded. The DNA pellet was air dried at 37 °C and suspended in 20 μl sterile distilled water. Total RNA was extracted from logarithmic, stationary and amastigote stages (infected THP1 cells) of *L. aethiopica*, and from uninfected THP1 cells using Trizol reagent as recommended by the manufacturer (Gibco-BRL).