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Evaluation of CAAX prenyl protease II of *Leishmania donovani* as potential drug target: Infectivity and growth of the parasite is significantly lowered after the gene knockout

Ruchika Bhardwaj [a], Mousumi Das [a,1], Shalini Singh [a,2], Adarsh Kumar Chiranjivi [a], Sitraraau Vijaya Prabhu [b], Sanjeev Kumar Singh [b], Vikash Kumar Dubey [a,*]

[a] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam 781039, India
[b] Computer Aided Drug Designing and Molecular Modeling Laboratory, Department of Bioinformatics, Alagappa University, Karaikudi, Tamil Nadu 630 004, India

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ABSTRACT

Prenylation pathway is responsible for post translational modification of various signal proteins, including proteins of Ras superfamily. CAAX prenyl proteases are known to be key players in prenylation pathway. In the current study, we have evaluated CAAX prenyl protease II as a possible drug target against *Leishmania donovani* parasite, the causative agent of visceral leishmaniasis. Gene knockout strategy was employed to target CAAX prenyl protease II and subsequent effects were studied. CAAX prenyl protease II knockout resulted in significant decrease in growth and infectivity.

1. Introduction

Leishmaniasis is one of most neglected tropical diseases. The causative agent of this disease is *Leishmania*, which is a dimorphic protozoan parasite. Leishmaniasis is associated with wide spectrum of clinical manifestations, ranging from self-recuperating cutaneous leishmaniasis to life threatening visceral leishmaniasis (Murray et al., 2005). Recent WHO statistics indicates the prevalence of this disease in 88 countries across 5 continents and 2 million new cases are occurring annually. The current drug scenario against this disease is not satisfactory due to poor efficacy, host toxicity, high cost and emergence of resistance (Croft et al., 2006). There is urgent need for drugs with higher efficacy, lower side effects and reasonable pricing. Target identification and validation are a key steps for any drug discovery process (Hughes et al., 2010).

It is well established that proteins undergo post-translational modification for their proper functional stimulation and regulation. Prenylation pathway is one such modification which is involved in maturation of various important signal proteins, like the ones belonging to Ras superfamily. These modified proteins further act as molecular switches for various signaling pathways that control important processes like cell proliferation, cell differentiation, membrane trafficking etc. Prenylation involves attachment of an isoprenoid group, i.e. 15C farnesyl or 20C geranylgaranyl group, to a cysteine residue by thioether linkage. Small GTP-binding proteins containing CAAX motif (C: cysteine, AA: aliphatic amino acid, X: any amino acid) at or near its carboxyl terminus are target molecules for prenylation (Zhang and Casey, 1996). CAAX prenyl protease is a key enzyme of prenylation pathway. There are two isoforms of this protease depending upon the substrate specificity and presence or absence of HEXXH (H: histidine, E: glutamate, X: any amino acid) conserved motif. CAAX prenyl protease I is an alpha-factor converting enzyme (AFC1) and possess HEXXH conserved motif (Boyartchuk et al., 1997; Schmidt et al., 2000). While on the other hand CAAX prenyl protease II is a Ras and yeast a-factor converting enzyme (RCE1), which lacks the HEXXH conserved motif (Dolence et al., 2000). Mislocalization of Ras proteins was observed in mouse embryonic fibroblast which lacked RCE1 (CAAX prenyl protease II) or ICMT (carboxyl methyl transferase) (Michaelson et al., 2005). Moreover some studies reported that RCE1 deficiency was lethal in late embryonic development in mouse hence indicating towards the physiological consequences of CAAX prenyl protease II (Kim et al., 1999). Further knockout of CAAX prenyl protease II in *Trypanosoma brucei* resulted in impairment of parasite growth (Gillespie et al., 2007). The crucial role of CAAX prenyl protease II is quite evident from these studies done on other organisms. Hence we chose to study

* Corresponding author.
E-mail address: vdubey@iitg.ernet.in (V.K. Dubey).
[1] Current address: Moffitt Cancer Center, Tampa, FL 33612, USA.
[2] Current address: University of Oxford, United Kingdom.

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the importance of this enzyme in *Leishmania donovani*, the causative agent of visceral leishmaniasis. We have earlier reported computational analysis on sequence and structural features of CAAX prenyl protease II from *Leishmania donovani* in comparison with the protein from human that showed low sequence similarity (24.12%) and significant differences at active site (Singh et al., 2016). The low sequence and active site structure similarity prompted us for selecting CAAX prenyl protease II of *Leishmania donovani* as potential drug target (Singh et al., 2016). In the current study, we have chosen CAAX prenyl protease II (LdBPK_262720) present in *Leishmania donovani* for evaluation as possible drug target by removing its expression from the parasite using homologous recombination. Effect of CAAX prenyl protease II knockout was studied in *Leishmania donovani*. Further complementation studies were also done to see reversal of the effects observed after CAAX prenyl protease II knockout.

2. Material and methods

2.1. Materials required

Gene specific primers of CAAX prenyl protease II, PCR clean up kit (Quiagen), Plasmid isolation kit (Sigma, USA), *Bam*HI, *Xho*I, *Sac*I, *Xma*I and *Eco*NI (NEB, USA), Gel extraction kit (Quiagen), T4 DNA ligase (NEB), Ampicilin, Hygromycin B, Puromycin (Himedia) and Geneticin (Gibco), Phleomycin (Sigma, USA), Genomic DNA isolation Kit (Quiagen), poly-L-lysine (Sigma, USA). Anti Ras antibodies produced in rabbit and anti-rabbit FITC conjugated secondary antibodies were purchased from Sigma-Aldrich, USA (Cat. No: SAB4301113 and F9887). Protein A purified rabbit-anti-CAAX prenyl protease II antibody was custom supplied Abgenex Pvt Ltd, India. Mouse-alpha tubulin antibodies (Cat. No. MA1-19162), goat-HRP-anti-rabbit IgG secondary antibodies (Cat. No. 656120) and rabbit-anti-mouse-IgG (H + L) peroxidase conjugated secondary antibodies (Cat. No. 31450) were purchased from ThermoFisher. The *Leishmania donovani* strain (BHU-1081) was obtained from Prof. Shyam Sunder, Banaras Hindu University, India. Macrophage cell line J774A, was taken from National Centre for Cell Science, Pune, India. The *Leishmania* expression vectors, used for preparing knockout cassettes as well as complementation vector were donated by Beverley Lab, Washington University Medical School, USA.

2.2. Parasite cell culture and maintenance

Leishmania donovani cells (MHOM/IN/2010/BHU1081) were obtained from Prof. Shyam Sundar (Banaras Hindu University, Varanasi, India). *Leishmania donovani* cells were maintained using the protocol already reported in earlier publications (Bhardwaj et al., 2016; Saudagar and Dubey, 2014; Das et al., 2013). In brief, *Leishmania* cells were cultured at 25 °C in complete M199 media. The M199 liquid media (pH 7.4) was supplemented with 15% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin. For the knockout selection process, parasites were grown in complete M199 media supplemented with respective antibiotics required for the selection process, i.e., Geneticin G418 (20 μg/ml) Hygromycin B (100 μg/ml), Puromycin (10 μg/ml) and Phleomycin (5 μg/ml) (Bhardwaj et al., 2016).

2.3. Preparation of molecular constructs for knockout as well as complementation

PCR amplification of 0.617 kbp upstream flanking region (5′UTR) and 0.448 kbp downstream flanking region (3′UTR) was done using *Leishmania donovani* genomic DNA as template. Overall strategy for gene knockout is shown in (Supplementary Fig. S1). Primers employed for this purpose were as follows: primer 1: 5′-CCTGCATTAGG CACAGACATGACGG-3′ and primer 2: 5′-CGCTCGAGGGAAGAACGGAT-3′, for 5′UTR amplification, primer 3: 5′-CGAGCTTCGGTACTATTATGAG-3′ and primer 4: 5′-CGGGAT CATGGAGGTGGAG-3′ for 3′UTR amplification. These amplified products were further cloned in *Leishmania* expression vectors pXG B1288 (NEO), pXG B3318 (HYG) and pXGB3325 (PAC) using *Xho*I-*Eco*NI and *Bam*HI-*Sac*I as restriction sites for 5′ UTR and 3′ UTR respectively. For complementation vector, 0.675 kbp CAAX prenyl protease II gene was amplified from *Leishmania donovani* genomic DNA using the following set of primers: primer 5: 5′-CCCCCGGGATGTGCTGCCTTGTCAG-3′ and primer 6: 5′-CGGGATCCTCAGTAGC GCAGCAGCG-3′. *Bam*HI and *Xma*I restriction sites were used to clone the gene in pXGB3324 (PHLEO) vector.

2.4. Generation of knockout strains of CAAX prenyl protease II and complementation

Removal of expression of CAAX prenyl protease II from the parasite was done by following gene knockout strategy based upon homologous recombination as shown in Supplementary Fig. S1 (Bhardwaj et al., 2016; Beverley and Clayton, 1993). In brief, the knockout cassettes were linearized by *Eco*NI digestion and were further transfected into *Leishmania* cells via electroporation. Approximately 10^7 cells/ml were washed twice with PBSG buffer (10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 145 mM NaCl and 2% glucose) followed by re-suspension in electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose). Further approximately 5-10 mg/ml linearized knockout cassettes were added and cell suspension was transferred to ice cold electroporation cuvette. After 10 min of incubation on ice, electroporation was performed (Exponential protocol: Voltage_450 V, Capacitance_500 F and the resulting time constant should be around 4.5 ms), again followed by incubation on ice. The cells were then incubated in complete M199 media at 25 °C for 24 h followed by transfer in antibiotic selection media. After first round of electroporation and selection, subsequent rounds of electroporation and selection were performed till we had all the three populations of knockout mutant viz., single knockout (CAAXII_SKO), double knockout (CAAXII_DKO) and triple knockout (CAAXII_TKO). The same method was employed for complemented cells with slight alterations. Here CAAXII_TKO cells were transfected with CAAX prenyl protease II gene containing pXG B3324 (PHLEO) vector. The selection was performed in M199 complete media containing 20 μg/ml of G418 for CAAXII_SKO cells, 20 μg/ml of G418 and 100 μg/ml Hygromycin B for CAAXII_DKO cells, 20 μg/ml of G418, 100 μg/ml Hygromycin B and 10 μg/ml Puromycin for CAAXII_TKO cells and finally 20 μg/ml of G418, 100 μg/ml Hygromycin B, 10 μg/ml Puromycin and 5 μg/ml Phleomycin for complemented cells. The selection was carried out for a week with continuous change in selection media. After the selection and confirmation of knockout as well as complemented cells, the cells were transferred to normal media for all subsequent experiments.

2.5. Confirmation of knockout cells

Preliminary confirmation of knockout was done by selection in Geneticin G418 (20 μg/ml) Hygromycin B (100 μg/ml) and Puromycin (10 μg/ml). The complemented cells were confirmed by selection in Geneticin G418 (20 μg/ml) Hygromycin B (100 μg/ml), Puromycin (10 μg/ml) and Phleomycin (5 μg/ml). To further substantiate the knockouts and complemented cells, western blot analysis was performed. Antibodies used against CAAX prenyl protease II were prepared in collaboration with Abgenex Pvt. Ltd. (Project ID: CP-49-15). Polyclonal antibodies were obtained by immunization of rabbits with synthetic peptide, which was derived from *Leishmania donovani* sequence followed by Protein A affinity purification. For western blot, cell lysates of wild type, CAAXII_SKO, CAAXII_DKO, CAAXII_TKO and complemented *Leishmania* cells were obtained by performing cell lysis and further procedure was followed as reported earlier (Bhardwaj et al., 2016). In brief, cells were harvested and washed in cold PBS (pH 7.4) followed by re-suspension in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 10 mM