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Research paper

Up-regulation of cytosolic tryparedoxin in Amp B resistant isolates of *Leishmania donovani* and its interaction with cytosolic tryparedoxin peroxidase

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ABSTRACT

Leishmania is a unicellular protozoan parasite which causes leishmaniasis, a neglected tropical disease. It possess a unique thiol metabolism comprising of several proteins among which, tryparedoxin (cTXN) and tryparedoxin peroxidase (cTXNPx), function in concert as oxidoreductases, utilizing trypanothione as a source of electrons to reduce the hydroperoxides produced by macrophages during infection. This detoxification pathway is unique and essential for the survival of *Leishmania*. Herein, we report the functional characterization of *Leishmania donovani* cTXN and its interaction with cTXNPx. The full length recombinant cTXN and cTXNPx proteins were purified in the native state and biochemical analysis showed that the cTXN-cTXNPx coupled system efficiently degraded hydrogen peroxide and tert-butyl hydroperoxide by transferring reducing equivalents from trypanothione. *In silico* investigation of the potential interaction between cTXN and cTXNPx proteins showed strong interaction of model structures with amino acids Ile109, Thr132, Glu107, Trp70, Trp39, Cys40 and His129 of Ld-cTXN and Thr54, Lys93, Arg128 and Asn152 of Ld-cTXNPx predicted to be involved in interaction. Moreover, co-purification, pull down assay and immunoprecipitation studies confirmed the interaction between Ld-cTXN and Ld-cTXNPx proteins. In addition, for the first time, we demonstrated at the translational level that Ld-cTXN protein is upregulated in Amp B resistant isolates accompanied by enhanced peroxidase activity, as compared to sensitive strains. Thus, our results show that Ld-cTXN and Ld-cTXNPx proteins acts in concert by physical interaction to form a strong peroxide stress detoxification system in *Leishmania* and their upregulation in Amp B resistant isolates imparts better stress tolerance, and hence fitter pathogens, as compared to sensitive strains.

1. Introduction

Leishmaniasis is a group of infectious diseases caused by protozoan parasites of the genus *Leishmania* and can infect several species of mammals including human. It is endemic in large areas of the world from tropical to the Mediterranean region affecting more than 1,300,000 people worldwide. Leishmaniasis is divided into four groups known as Cutaneous, Mucocutaneous, Visceral leishmaniasis (VL, Kala-azar) and Post Kala-azar Dermal Leishmaniasis based on the site of infection in their mammalian hosts. Approximately 90% of the VL cases in the world are reported from Bangladesh, India, Brazil, Sudan, and Nepal. The global incidence

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and prevalence rate of VL are 0.5 and 2.5 million per year [1], mainly affecting developing countries, and it poses a major health problem in Bihar which accounts for nearly 90% of the total VL cases in India [2]. VL is the most severe form of disease, ranked next to malaria among human protozoan infections, and is generally fatal in the majority of untreated cases. The available first line drug sodium stibogluconate (SAG) for the treatment of VL is no more preferred because 65% infected cases are found to be resistant against the drug. The second line drug Amphotericin B (Amp B) also show relapse and/or resistance [3,4] but clinical resistance to Amp B in *Leishmania donovani* is very low, although few cases have been reported recently in India [5,6]. Moreover, the available drugs for treatment of leishmaniasis are not satisfactory because of high toxicity, poor efficacy or loss of effectiveness due to development of drug resistance after prolonged use [7,8]. In view of the increase in drug resistance combined with inadequate drug efficacy and safety, there is an urgent need of new and highly specific drug or vaccine to replace or complement the currently available drugs [9]. Clues for rational drug design can be gained by targeting the metabolic pathways that are crucial for parasite viability or infectivity, and either absent or differ significantly from those found in their mammalian host [10]. The thiol metabolism or their components have been considered as drug target because this pathway is missing in their mammalian host. Moreover, trypanothione reductase and tryparedoxin peroxidase proteins are secreted by the parasites in the culture medium and may serve as potential vaccine candidates [11,12].

Most of the parasites including *Leishmania* spp., are more susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS) as compared to their hosts [7]. To establish infection and evade the potential cell damage from ROS and RNS, parasites have developed different antioxidant defense systems [13]. Trypanosomatids, including *Leishmania*, possess a unique dithiol trypanothione ($T(SH)_2$) based antioxidant system, in contrast to their host which rely on glutathione and catalase enzyme. $T(SH)_2$, is synthesized by trypanothione synthetase (TryS) from two molecules of glutathione and one molecule of spermidine, and is maintained in the reduced form by trypanothione reductase (TryR) using NADPH as electron donor [6,14,15]. The reduced $T(SH)_2$ is involved in many enzymatic reactions of different metabolic pathways, such as, synthesis of DNA precursors, detoxification of oxidants, metals and drugs, as electron donor to oxidized glutathione, dihydroascorbic acid, and tryparedoxin/tryparedoxin peroxidase couple for the reduction of hydrogen peroxide to water [14,16]. One of the major biological functions of the $T(SH)_2$ pathway is to regulate oxidative and nitrosative stress by shuttling reducing equivalents from NADPH to hydroperoxides and peroxynitrites [17,18]. The thiol metabolic pathway has been recognized as a potential drug target for anti-leishmanial drug development since it is absent in the host and most of its components are essential for the parasites survival [14,19,20].

Tryparedoxin (TXN) is a low molecular-mass dithiol protein of thioredoxin oxidoreductase super family and possesses a WCPPC motif near the catalytic pocket [21,22]. The TXN exists in two isoforms, TXNI and TXNII, and these isoforms are localized in the cytosol and mitochondria, respectively. The TXN are similar in structure to thioredoxins; both having a core of five-stranded β-sheets surrounded by four α-helices [23,24]. However, TXN contains a unique insertion of 24–36 amino acids between α-2 and β-5 domains, which contributes to the slightly higher molecular mass of TXNs (~16 kDa) as compared to thioredoxins (~12 kDa) [24,25]. Tryparedoxin peroxidase (TXNPx) performs an analogous function to glutathione peroxidase of mammals. It belongs to the 2-cysteine peroxiredoxin family, and more than one isoform of TXNPx has been reported which are localized in the cytosol and mitochondria [26]. A unique feature of the cytosolic TXNPx (cTXNPx) is that it uses reducing equivalents of cytosolic tryparedoxin (cTXN) derived from $T(SH)_2$, in contrast to other eukaryotes which utilize glutathione (GSH) [26–28]. TXN and TXNPx proteins are generally present in other trypanosomatids and they are highly conserved in all *Leishmania* species [29,30]. These proteins play roles in defense against chemical and oxidative stress, i.e., reduction of hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH) into water and alcohol, respectively. These enzymes also have a key role in DNA biosynthesis, DNA replication, and actively participate in ROS regulation. TXN transfers electrons to ribonucleotide reductase (RR) for the synthesis of deoxyribonucleotides which is the precursor for DNA synthesis. Another isoform of TXN present in the mitochondria has been reported to be involved in the transfer of electrons to the transcription factor, the universal minicircle sequence binding protein (UMSBP), and a monothiol glutaredoxin via peroxidase. The UMSBP redox state is supposed to be maintained by TXN-TXNPx pair and it participates in the initiation of replication of kinetoplast DNA. The combined action of cTXN, cTXNPx and TryR helps in the maintenance of a low concentration of H_2O_2 and together they constitute the redox metabolism of the parasites, vital for their survival [13]. In addition to ROOH, TXNPx have also been shown to decompose peroxynitrite by distinct reactions in bacteria [17] and *Leishmania chagasi* [31] in the presence of TXN. Recently, the cTXN and cTXNPx of *Leishmania major* were crystallized and Surface Plasmon Resonance (SPR) based analysis of their electrostatic surface potential revealed interaction between them [32]. Both the cTXN and cTXNPx are homodimers but in contrast to cTXN, cTXNPx is organized in pentamers $(\alpha_2)_5$ and the resulting quaternary structure corresponds to a toroidal decamer form composed of two adjacent asymmetric units [32]. The structure-based high throughput docking to the available crystal structures of *L. major* cTXNPx identified several novel compounds as enzyme inhibitors, such as *N,N*-disubstituted 3-aminomethyl quinolone derivatives, which are non-covalently bound to cTXNPx and may serve as potential drug target against leishmaniasis [33].

Previously, TXN knockout experiments in *Leishmania infantum* [34] and RNAi experiments in *Trypanosoma brucei* [35] demonstrated the essentiality of this protein in the parasites survival and antioxidant metabolism. In *Trypanosoma cruzi*, two genes encoding for TXN have been identified and functionally characterized viz. cytosolic TXNI and mitochondrial TXNII [19,36–38]. TXNI differs from TXNII in that, former has a central insertion of 15 amino acids whereas latter contains an extra C-terminal tail of 26 amino acids. Recently, proteomic studies by incubating mutant of TXNI and TXNII protein with *T. cruzi* lysate proteins identified fifteen [37] and sixteen [39] interacting proteins, respectively, isolated as heterodisulfide complexes, which are involved in wide range of cellular processes. However, such interactome of cytosolic TXN of *L. donovani* has not yet been explored.

Here, for the first time in *L. donovani*, we show that cTXN protein interacts with cTXNPx to form a stable complex by *in silico* study, pull-down and immunoprecipitation assay. Further, we demonstrate that purified cTXN transfers reducing equivalent to cTXNPx, allowing it to catalyze the reduction of H_2O_2 or (ROOH) into water or alcohol, respectively, indicating its functional role under oxidative stress conditions. In addition, the cTXN protein level was found to be upregulated in Amp B resistant isolates of *L. donovani* indicating its potential role in acquisition of drug resistance.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals of analytical grade were purchased and used from Sigma–Aldrich, Amresco (USA), Merck, and USB (USA)