Critical Importance of the *de novo* Pyrimidine Biosynthesis Pathway for *Trypanosoma cruzi* Growth in the Mammalian Host Cell Cytoplasm

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Footnote 1:

Abbreviations: CPSII, carbamoyl-phosphate synthetase II; LIT, liver infusion tryptose; DHOD, dihydroorotate dehydrogenase; MOI, a multiplicity of infection

ABSTRACT

The intracellular parasitic protist *Trypanosoma cruzi* is the causative agent of Chagas disease in Latin America. In general, pyrimidine nucleotides are supplied by both *de novo* biosynthesis and salvage pathways. While epimastigotes—an insect form—possess both activities, amastigotes—an intracellular replicating form of *T. cruzi*—are unable to mediate the uptake of pyrimidine. However, the requirement of *de novo* pyrimidine biosynthesis for parasite growth and survival has not yet been elucidated. Carbamoyl-phosphate synthetase II (CPSII) is the first and rate-limiting enzyme of the *de novo* biosynthetic pathway, and increased CPSII activity is associated with the rapid proliferation of tumor cells. In the present study, we showed that disruption of the *T. cruzi CPSII* gene significantly reduced parasite growth. In particular, the growth of amastigotes lacking the *CPSII* gene was severely suppressed. Thus, the *de novo* pyrimidine pathway is important for proliferation of *T. cruzi* in the host cell cytoplasm and represents a promising target for chemotherapy against Chagas disease.

Keywords: *Trypanosoma cruzi*, pyrimidine biosynthesis pathway, carbamoyl-phosphate synthetase II, gene targeting, cell growth

INTRODUCTION

The parasitic protist *Trypanosoma cruzi* is the causative agent of Chagas disease [1]. The parasitic life cycle comprises 2 phases (the insect and mammalian stages) and includes 3 developmental forms—epimastigotes, trypomastigotes, and amastigotes [2]. Within the insect vector, the reduviid bug, epimastigotes replicate and transform into metacyclic trypomastigotes by a process termed metacyclogenesis. Non-proliferating metacyclic trypomastigotes invade the mammalian host and subsequently transform into amastigotes in a wide variety of nucleated cells. Intracellular amastigotes multiply by binary fission and then transform back into bloodstream trypomastigotes, which are released into the circulation after host cell disruption.

Pyrimidine is an essential component of nucleic acid structure. Thus, the biosynthesis of pyrimidine is a vital biological process, which is achieved by both *de novo* synthesis and salvage pathways. Although both pathways have been shown to operate in *T. cruzi*, the parasite has only limited salvage activity. It has been reported that *T. cruzi* preferentially takes up more pyrimidine bases and nucleosides than nucleotides [3]. However, the host cell cytoplasm contains mainly nucleotides [4], which are not efficiently salvaged by *T. cruzi*. In addition, the intracellular amastigote lacks uracil phosphoribosyltransferase and uridine kinase enzymatic activities, the latter of which is not found in the *T. cruzi* genome [5]. Therefore, the *de novo* pyrimidine biosynthesis pathway may be important for *T. cruzi* growth in mammalian hosts.

The *de novo* pyrimidine biosynthetic pathway consists of a 6-enzyme cascade that catalyzes the formation of uridine 5′-monophosphate [6]. The first enzyme in the pathway

is carbamoyl-phosphate synthetase II (CPSII, EC 6.3.5.5), which generates carbamoyl-phosphate from L-glutamine, bicarbonate, and 2 ATP molecules. Moreover, CPSII is a key regulatory enzyme of *de novo* pyrimidine biosynthesis, and increased CPSII activity is associated with the rapid proliferation of tumor cells [7].

In the present study, we established *T. cruzi* lacking the *CPSII* (*TcCPSII*) gene in order to determine the physiological relevance of *de novo* pyrimidine biosynthesis. By analyzing the mutant parasites, we found that the *de novo* pyrimidine pathway is important for parasitic growth in the host cell.

MATERIALS and METHODS

T. cruzi culture

T. cruzi epimastigotes (Tulahuen strain) were routinely subcultured on a weekly basis in liver infusion tryptose (LIT) medium (No. 1029, ATCC medium formulations) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 μg/ml hemin (Sigma-Aldrich, Japan) in tightly capped 25-cm^2 culture flasks at 27°C. The mammalian stages of *T. cruzi* were maintained in HeLa cells, as previously described [8]. In addition, we used mouse embryonic fibroblast 3T3-SWISS albino cells (Health Science Research Resources Bank, Tokyo, Japan), which were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Japan) supplemented with 10% FBS. For experiments, 3T3-SWISS cells were infected with metacyclic trypomastigotes at a multiplicity of infection (MOI) of 0.26. The infected cells were washed with PBS 24 h after infection to remove free parasites and cultured for a further 3 days. Multiple infections of a single host

cell by metacyclic trypomastigotes were confirmed as negligible under the experimental conditions employed. Infected *T. cruzi* amastigotes within host cells were stained with Diff-Quik solution (Sysmex, Kobe, Japan) and detected microscopically as previously described [8].

Metacyclogenesis

Metacyclogenesis was induced as previously described [9]. Epimastigotes from the late logarithmic phase were collected by centrifugation, suspended at a density of 1.0×10^7 cells/ml in 80% (v/v) RPMI-1640 medium and 20% (v/v) Grace's insect medium and incubated at 27°C. Under these conditions, epimastigotes adhered to the surface of the plastic culture flask, and were released only once they enter metacyclogenesis [10]. For counting of metacyclic trypomastigotes, parasites that appeared in the culture supernatant were stained with Giemsa stain, and their morphology was evaluated by light microscopy according to the relative kinetoplast-nucleus position [10; 11; 12]. The efficacy of metacyclogenesis was evaluated by the ratio of epimastigotes to trypomastigotes in the culture supernatant 72 h after induction.

To examine the infectivity of host cells by metacyclic trypomastigotes, the remaining epimastigotes were killed by adding untreated (not heat-inactivated) FBS, as previously described [13]. The trypomastigotes were collected by centrifugation at 800 *g* for 5 min in 15-ml polypropylene tubes to remove cell debris. The resulting supernatant was centrifuged at 1,500 *g* for 10 min at 4°C, and the pellet containing trypomastigotes was washed 3 times with 10 ml of DMEM by repeated suspension and centrifugation. The purified

trypomastigotes were counted on an improved Neubauer hemocytometer and used for experiments.

Generation of the knockout DNA cassettes for *TcCPSII*

We constructed 2 gene-knockout cassettes comprising the 5′ flanking region of *TcCPSII*, the neomycin phosphotransferase II gene (neo^R) or hygromycin B phosphotransferase gene (hyg^R), and an internal sequence from the *TcCPSII* gene (nucleotides +1,000 to +2,000) to allow disruption of the *TcCPSII* loci by homologous recombination (Fig. 1A). The knockout cassettes were constructed using a MultiSite Gateway® Three-Fragment Vector Construction Kit (Invitrogen) [14]. The pDEST/TcCPSII_Neo^R plasmid was constructed by amplifying the 5′ flanking sequence of *TcCPSII* (0.41 kb) using a primer set (sense, 5′-GGGGACAACTTTGTATAGAAAAGTTGGAATTCGCGTCTTCCTTTTTTTCTTCCTTTTCTTTT-3′ and antisense, 5′-GGGGACTGCTTTTTTGTACAAACTTGTGTTTTACTTTTTTATGTTTTGTGTTACTG-3′; the *Eco*RI site is underlined), KOD-Plus-Neo (TOYOBO), and *T. cruzi* Tulahuen genomic DNA as the template and was subsequently cloned into the entry vector (pDONR P4-P1R) via a BP reaction. Similarly, the coding region of *TcCPSII* (+1,000 to +2,000 bp) was amplified using a primer set (sense, 5′-GGGGACAGCTTTCTTGTACAAAGTGGTTCTCTGTACAGTTTCATCCG-3′ and antisense, 5′-GGGGACAACTTTGTATAATAAAGTTGGAATTCAGACGTGCATTAACCTCAATT-

3′) and cloned into pDONR P2R-P3. The *neo*^{R} gene was amplified using a primer set (sense, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGATTGAACAAGATGGATT-3′ and antisense, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCAGAAGAACTGGTCAAGAA-3′) and pTREX DNA as the template and cloned into entry vector pDONR 221. In order to generate the final plasmid, the 3 recombinant plasmids were subsequently transferred to a destination vector, pDEST R4-R3, according to the manufacturer's instructions. Finally, the knockout DNA cassette was excised from the plasmid backbone with *Eco*RI and used for electroporation.

To construct the pDEST/TcCPSII_Hyg^{R} plasmid, a 0.41-kb 5′ flanking sequence from *TcCPSII* was amplified using *T. cruzi* Tulahuen genomic DNA, a primer set (sense, 5′-GGGGACAACTTTGTATAGAAAAGTTGCAGCTGGCGTCTTCCTTTTTTTCTTCCT TTTCTTTT-3′; the *Pvu*II site for digestion is underlined and antisense, 5′-GGGGACTGCTTTTTTGTACAAACTTGTGTTTTACTTTTTTATGTTTTGTGTTACT G-3′) and KOD-Plus-Neo (TOYOBO) and cloned into the entry vector (pDONR P4-P1R) via a BP reaction. Similarly, a region of the *TcCPSII* ORF sequence (+1,000 to +2,000) was amplified from *T. cruzi* Tulahuen genomic DNA using a primer pair (sense, 5′-GGGGACAGCTTTCTTGTACAAAGTGGTTCTCTGTACAGTTTCATCCG-3′ and antisense, 5′-GGGGACAACTTTGTATAATAAAGTTGCAGCTGAGACGTGCATTAACCTCAATT-3′; the *Pvu*II site for digestion is underlined) and cloned into pDONR P2R-P3. Using the pTEX-derived plasmid, p72hyg72 [15; 16], as a template, the *hyg*^{R} gene was amplified with

a primer set (sense,

5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAAAGCCTGAACTCACC-3′

and antisense,

5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTTCCTTTGCCCTCGGACGAGT-3′)

and cloned into entry vector pDONR 221. The 3 entry clones were subsequently mixed with a destination vector (pDEST R4-R3) to generate the final plasmid by an LR reaction. The knockout DNA cassette was released from the plasmid backbone by *Pvu*II digestion.

Transformation of *T. cruzi* using knockout DNA cassettes

About 1×10^7 early log-phase epimastigotes were suspended in 100 µl of Amaxa Basic® Parasite Nucleofector Kit 2 solution (Lonza). Transformation of the parasites was carried out using 10 µg of the knockout DNA cassette and the "U-033" program of an Amaxa Nucleofector Device (Lonza). Stable transformants were selected by incubating cells for 30–45 days in LIT medium containing 0.25 mg/ml G418 (for single-gene knockout [SKO] with neo^R) or 0.25 mg/ml G418 plus 0.25 mg/ml hygromycin B with 0.2 mM uracil (for double-gene knockout [DKO] with neo^R and hyg^R) and cloned by limiting dilution. Integration of the knockout cassette into the precise locus of the *TcCPSII* gene was confirmed by PCR using DNA cassette-specific sense primers (5′-ATCGCCTTCTTGACGAGTTCT-3′ for neo^R and 5′-ACTCGTCCGAGGGCAAAGGAA-3′ for hyg^R) and the *TcCPSII*-specific antisense primer (5′-CATTGTTGTCTTGGTGACCCC-3′; +2101 to +2122 bp).

Real-time PCR

Total RNA was isolated from wildtype (WT), *CPSII* SKO, and *CPSII* DKO epimastigotes using Agilent Total RNA Isolation mini kits (Agilent Technologies, Santa Clara, CA). Subsequently, cDNA was prepared by reverse transcription using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Bio Systems, Foster City, CA) and the following primers (*TcCPSII*-specific sense, 5′-TGGCCTTTTTATTTCCAACG-3′ and antisense, 5′-CGATGGCCGTACTTCATCTT-3′; *T. cruzi* beta tubulin-specific sense, 5′-TTTGTCGGCAACAACACCTG-3′ and antisense, 5′-CTAGTACTGCTCCTCCTCGT-3′).

RESULTS and DISCUSSION

Construction of *TcCPSII*-knockout *T. cruzi*

T. cruzi is a diploid organism [17]. We previously demonstrated that the *TcCPSII* gene is a single-copy gene in *T. cruzi* Tulahuen and occurs at 2 gene loci per cell [6]. Thus, we aimed to generate *TcCPSII*-knockout parasites in order to investigate the physiological relevance of the *de novo* pyrimidine biosynthesis pathway in *T. cruzi*.

We constructed 2 gene knockout cassettes comprising the 5′ flanking region of *TcCPSII*, the neomycin phosphotransferase II gene (neo^R) or hygromycin B phosphotransferase gene (hyg^R), and the coding region of the *TcCPSII* gene (+1,000 to +2,000 bp) to allow disruption of the *TcCPSII* loci by homologous recombination (Fig. 1A).

Integration of the knockout cassettes was confirmed by PCR using a sense primer specific for the resistance marker gene and an antisense primer specific for a region outside the 3′ cassette (Fig. 1B and 1C). For cloning of the DKO parasites, the culture medium was supplemented with uracil in order to complement the defect due to loss of the *CPSII* gene.

We analyzed the expression levels of *TcCPSII* transcripts in WT, SKO, and DKO parasites by real-time RT-PCR (Fig. 1D). A reduction in expression levels of approximately 50% was observed for the SKO parasites, which was directly proportional to the copy number of the functional *TcCPSII* gene. Moreover, transcription of *TcCPSII* in DKO parasites was at trace levels, indicating that DKO parasites are *TcCPSII*-null mutants.

Physiological importance of TcCPSII for the growth of *T. cruzi* epimastigotes

We investigated the physiological importance of *de novo* pyrimidine biosynthesis in epimastigotes by comparing the growth of WT, SKO, and DKO epimastigotes in LIT medium supplemented with 10% FBS. The parasite growth curves for 9 d of cultivation are shown in Fig. 2. While the growth rates were almost identical for the first 6 d of cultivation, significant suppression of the growth of both SKO and DKO parasites was observed after 9 d of cultivation. Notably, the growth of the DKO parasites was more severely impaired than the growth of SKO parasites.

The addition of uracil (final concentration of 500 μM) rescued the growth defect of DKO epimastigotes to a level comparable to that of SKO parasites between days 7 and 9 (Fig. 2). Therefore, since the LIT medium appeared to contain a sufficient amount of pyrimidine precursors to support the growth of *CPSII*-knockout parasites during the early

stages of cultivation, these results suggests that epimastigotes preferentially consume pyrimidine precursors in the medium during the first 6 d of cultivation and subsequently depend on *de novo* pyrimidine biosynthesis due to the absence of precursors after 7 d of cultivation.

To further confirm that the growth defect of DKO parasites was due to the lack of CPSII activity, we attempted to rescue parasites by overexpression of TcCPSII. The DKO and WT epimastigotes were transformed using a trypanosomal expression plasmid, pTREX [18], which carries the *TcCPSII* gene. However, we were unable to obtain transgenic parasites neither for the DKO nor for the WT background, suggesting that overexpression of TcCPSII is highly toxic for the parasites. Furthermore, we found that a high concentration of uracil (> 500 μM) impaired the growth of parasites (data not shown). Therefore, as previously reported [3], both *de novo* pyrimidine biosynthesis and salvage pathways were shown to be physiologically active in epimastigotes. Moreover, we found that CPSII is required for parasite growth under culture conditions of insufficient salvage substrates.

During the life cycle of *T. cruzi*, epimastigotes transform into metacyclic trypomastigotes, which represent a transmission stage from the insect vector to mammalians. Metacyclogenesis is also inducible in conditioned medium lacking pyrimidine precursors [9]. Thus, we examined whether a pyrimidine supply is required for transformation of *CPSII*-SKO and DKO parasites. We compared the efficacy of metacyclogenesis of SKO, DKO, and WT parasites and found no significant difference between the groups (data not shown). Therefore, these results suggest that nucleotide

synthesis is not critical for the process of metacyclogenesis.

Physiological importance of TcCPSII for *T. cruzi* amastigote growth in the host cell

In general, pyrimidine *de novo* biosynthesis is particularly important in rapidly growing cells [7]. Since *T. cruzi* amastigotes are only proliferative in mammalian hosts, we examined whether CPSII and the *de novo* pyrimidine biosynthesis pathway are required for the growth of amastigotes.

We infected 3T3-SWISS fibroblast cells with metacyclic trypomastigotes from WT, SKO, or DKO parasites at an MOI of 0.26, and the average number of intracellular amastigotes per infected cell was compared between the groups. Multiple infections of a single host cell by metacyclic trypomastigotes were negligible under the experimental conditions employed (data not shown). The amastigote counts were significantly reduced for SKO and DKO parasites in a dose-dependent manner (Fig. 3), suggesting that *de novo* pyrimidine biosynthesis is more important for amastigotes than epimastigotes.

Extracellular amastigotes incorporate pyrimidine bases and nucleosides to a significantly lesser extent compared with epimastigotes and trypomastigotes [3]. In addition, the average concentrations of uridine in mammalian tissues and plasma are estimated to be of the micromolar order [4; 19]. Therefore, amastigotes are likely to rely on *de novo* pyrimidine biosynthesis. We observed that DKO amastigotes appeared to replicate approximately 2 times after 4 days of cultivation. These results indicate that the concentrations of intracellular pyrimidine precursors are insufficient to support amastigote replication, resulting in the dependence on *de novo* pyrimidine biosynthesis. Moreover, our

observations are in good agreement with a study on another intracellular parasite, *Toxoplasma gondii*, which also has limited pyrimidine salvage ability [20]. Indeed, the *CPSII*-null mutant of this apicomplexan parasite was only able to replicate inside the host cell in culture medium supplemented with >200 μM uracil as a pyrimidine source.

We have previously shown that disruption of 2 of the 3 loci for the dihydroorotate dehydrogenase (*DHOD*) gene, encoding the fourth enzyme of the *de novo* pyrimidine biosynthesis pathway, led to the gradual death of *T. cruzi* epimastigotes, which were no longer viable 2 weeks later [16]. Furthermore, the addition of uridine, cytidine, and thymidine (200 μM each) to the medium did not enable growth of *DHOD* DKO epimastigotes. While these findings differ from the phenotype of *CPSII* DKO epimastigotes, the phenotypic difference between *CPSII* and *DHOD* KO parasites may be attributed to the coupled reaction of DHOD. In *T. cruzi*, catalysis of the oxidation of dihydroorotate by DHOD is coupled with the reduction of fumarate to succinate. In addition, this reaction is reversible. Therefore, it is likely that the fumarate-reductase activity of DHOD—as well as succinate production by its reverse reaction—contributes to maintenance of the redox balance in epimastigotes. Moreover, *T. cruzi* DHOD may use not only dihydroorotate but also other unidentified substrates as electron donors/acceptors for succinate/fumarate metabolism.

Thus, we conclude that the *de novo* pyrimidine biosynthesis pathway is critical for intracellular replication of *T. cruzi*. Our findings provide insight into CPSII and the pyrimidine metabolic pathway, which represents a promising target for chemotherapy. The screening of chemical compound libraries by using recombinant enzymes and bioassay

systems may facilitate the identification of seed compounds for drug development against Chagas disease.

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FIGURE LEGENDS

Figure 1. Production of a *T. cruzi CPSII*-null mutant

(A) Diagram of the *TcCPSII* locus and neomycin- and hygromycin-resistance gene cassettes containing 5′-upstream (−0.41 kb) and 3′-downstream (+1.0 kb) sequences corresponding to the downstream region (+1 kb to +2 kb) of the *TcCPSII* open reading frame (ORF). (B) Schematic representation of *TcCPSII* loci in the WT, single-gene knockout (SKO), and double-gene knockout (DKO) parasites. (C) PCR analysis of genomic DNA from clones of the WT, SKO, and DKO parasites confirmed disruption of 1 locus of the *TcCPSII* gene in SKO parasites and disruption of the 2 loci in DKO parasites, respectively. (D) Real-time PCR analysis of *TcCPSII* mRNA expression levels in WT, SKO, and DKO epimastigotes. The data shown are expressed as fold change and represent the mean ± SD of 3 independent experiments.

Figure 2. Physiological role of CPSII in the growth of *T. cruzi* epimastigotes

A total of 1×10^6 WT, SKO, or DKO epimastigotes were grown in liver infusion tryptose (LIT) medium, and the average number of parasites from 3 independent experiments is shown. DKO + uracil represents DKO parasites grown in the presence of 500 μM uracil.

Figure 3. Physiological role of CPSII in the growth of *T. cruzi* amastigotes

A total of 1.5×10^5 3T3-SWISS Albino cells were infected with 4×10^4 WT, SKO, or DKO metacyclic trypomastigotes (MOI, 0.26) and cultured for 4 days. The average number of parasites per infected cell is shown, and data represent the mean ± SD of 3 independent

experiments.