International Journal for Parasitology 39 (2009) 533–539

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Genetic identification of essential indels and domains in carbamoyl phosphate synthetase II of *Toxoplasma gondii*

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ARTICLE INFO

Article history:
Received 3 June 2008
Received in revised form 16 September 2008
Accepted 17 September 2008

Keywords:
Toxoplasma gondii
Carbamoyl phosphate synthetase II
Complementation
Essential indels
Mutations

ABSTRACT

New treatments need to be developed for the significant human diseases of toxoplasmosis and malaria to circumvent problems with current treatments and drug resistance. Apicomplexan parasites causing these lethal diseases are deficient in pyrimidine salvage, suggesting that selective inhibition of de novo pyrimidine biosynthesis can lead to a severe loss of uridine 5′-monophosphate (UMP) and thymidine 5′-monophosphate (dTMP) pools, thereby inhibiting parasite RNA and DNA synthesis. Disruption of *Toxoplasma gondii* carbamoyl phosphate synthetase II (CPSII) induces a severe uracil auxotrophy with no detectable parasite replication in vitro and complete attenuation of virulence in mice. Here we show that a CPSII cDNA minigene efficiently complements the uracil auxotrophy of CPSII-deficient mutants, restoring parasite growth and virulence. Our complementation assays reveal that engineered mutations within, or proximal to, the catalytic triad of the N-terminal glutamine amidotransferase (GATase) domain inactivate the complementation activity of *T. gondii* CPSII and demonstrate a critical dependence on the apicomplexan CPSII GATase domain in vivo. Surprisingly, indels present within the *T. gondii* CPSII GATase domain as well as the C-terminal allosteric regulatory domain are found to be essential. In addition, several mutations directed at residues implicated in allosteric regulation in *Escherichia coli* CPS either abolish or markedly suppress complementation and further define the functional importance of the allosteric regulatory region. Collectively, these findings identify novel features of *T. gondii* CPSII as potential parasite-selective targets for drug development.

1. Introduction

Current chemotherapeutic treatments of human infections with apicomplexan parasites selectively target distinguishing aspects of essential parasite enzymes or essential biological functions. While current therapies are often effective, challenges are presented by the development of drug resistance as well as problems associated with long-term treatment regimes. Consequently, development of new drug treatments exhibiting good efficacy, low toxicity, selectivity to parasite but not host, and delayed development of widespread drug resistance is a high priority. Apicomplexan parasites such as *Plasmodium falciparum* and *Toxoplasma gondii* rely on a de novo pyrimidine synthesis pathway that is an excellent target for chemotherapy to inhibit both RNA and DNA synthesis (Fox and Bzik, 2002; Chaudhary et al., 2007; Fox et al., 2007; Painter et al., 2007). Genetic disruption of carbamoyl phosphate synthetase II (CPSII) in *T. gondii* induces a severe uracil auxotrophy as demonstrated by the complete absence of parasite replication in vitro and extreme attenuation of virulence in murine infection, even in severely immune-deficient IFNg$^{-/-}$ mice (Fox and Bzik, 2002). CPSII knock-out strains such as *cps1-1* invade host cells but do not replicate in vivo (Fox and Bzik, 2002). Strain *cps1-1* possesses a unique and potent ability to elicit strong and long-lasting host protective immune responses (Fox and Bzik, 2002; Ling et al., 2006; Shaw et al., 2006; Dzierszinski et al., 2007; Zhao et al., 2007; Dzierszinski and Hunter, 2008; Wilson et al., 2008).

CPS catalyses the production of carbamoyl phosphate from two ATP molecules, glutamine, bicarbonate and water. In most organisms carbamoyl phosphate is used as the precursor molecule for biosynthesis of uridine monophosphate (UMP) and arginine (Beckwith et al., 1962; Jones, 1980; Davis, 1986). Surprisingly, parasites from the phylum Apicomplexa (*Plasmodium* sp. and *T. gondii*) are natural arginine auxotrophs and the parasite CPSII is the first committed step in pyrimidine biosynthesis (Fox et al., 2004).

Escherichia coli CPS consists of a large subunit (CPS domains) with binding sites for substrates and allosteric effectors, and an associated small subunit glutamine amidotransferase (GATase) belonging to the Class I amidotransferase superfamily which catalyses the hydrolysis of glutamine to glutamate and ammonia (Beckwith et al., 1962; Foley et al., 1971; Raushel et al., 1978; Thoden et al., 1999a). In contrast, the eukaryotic CPSII has an N-terminal GATase that is fused to the CPS polypeptide, as well as possessing fused C-terminal domains that catalyse subsequent steps in pyrim-

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0020-7519/$34.00
doi:10.1016/j.ijpara.2008.09.011

idine biosynthesis (Jones, 1980; Davis, 1986; Davidson et al., 1993). Protozoa, which include medically important parasites (*Plasmodium*, *Toxoplasma*, *Babesia*, *Trypanosoma*, *Leishmaina*), possess a single atypical and simplified eukaryotic CPSII comprising an N-terminal GATase fused to CPS via an atypically short linker but do not possess fused C-terminal domains that catalyse subsequent steps in pyrimidine biosynthesis (Aoki et al., 1994; Flores et al., 1994; Chansiri and Bagnara, 1995; Gao et al., 1998, 1999; Nara et al., 1998; Fox and Bzik, 2003). Because these subsequent enzyme activities of the de novo pyrimidine pathway are not directly fused to the C-terminus of protozoan forms of CPSII, we could easily engineer a functional CPSII minigene to evaluate genetic complementation of *T. gondii* CPSII function in vivo.

While the structure, function and mechanisms of CPS catalysis and regulation have been well characterised for *E. coli* CPS (Thoden et al., 1997, 1999b; Mora et al., 1999; Fresquet et al., 2000; Huang and Raushel, 2000a,b,c; Miles and Raushel, 2000), the larger CPSII polypeptides of eukaryotes are not yet well characterised at both the functional and structural level (Davidson et al., 1993; Graves et al., 2000; Fox and Bzik, 2003; Simmons et al., 2004; Kothe et al., 2005). Here, we report functional complementation of uracil auxotrophy in *T. gondii* based on CPSII minigenes. The development of an efficient system for complementation of uracil auxotrophy provides a new genetic model for positive selection and enables a direct genetic dissection of functional domains in a eukaryotic CPSII enzyme in vivo. Our results identify unique indels present in apicomplexan CPSII as distinct, functionally important, domains which may potentially serve as novel parasite-selective drug targets.

2. Materials and methods

2.1. Plasmid construction

A functional CPSII minigene encoding the authentic 1,687 amino acids of CPS was constructed by sequential coupling of defined cDNA segments generated by reverse transcriptase-PCR. Firstly, a 1,829-bp cDNA for the N-terminal GATase domain of CPSII was amplified from polyA+ mRNA from the RH strain (5′-ACTAGTG GTGATGACGACGACAAGATGCCTCACAGTGGAGGGC-3′ and 5′-GAT-ATCCACGTGTCGCGGCCGCGCTCTC-3′). The 1,829 bp cDNA was introduced (SpeI/EcoRV) into plasmid pET41b (SpeI/XhoI-blunted). Next an N-terminal section of the CPS domain cDNA comprising bp 1,829 to bp 3,532 was generated (5′-GAGAGCGCGGCCGCGAC-3′ and 5′-CACGTGGAGGCGAGACGTCGTCGTC-3′) and fused to the GATase domain (NotI/PmlI). The remainder of the CPS domain was constructed by amplifying two cDNA segments, bp 3,003–4,097 and bp 4,097–5,064 (5′-AGTACTTGATGAATTCACCG-3′ and 5′-TTTCTGCGAGATCTTCTTCACG-3′, 5′-GCGTGAAGAAGATCTCGCAG-3′ and 5′-ATCGATCACGTGATTTTTGAGGCCAGTATTCATCC-3′, respectively), and then the two C-terminal segments were fused in plasmid PCR4TOPO (EcoRI/BglII). Finally, the C-terminal section of CPS was fused with the N-terminal section in plasmid PET41b (EcoRI/PmlI) and the complete 5,064 bp CPSII minigene coding sequence was determined to verify authenticity.

The 5′ untranslated region (UTR) and 3′ UTR were amplified from RH genomic DNA. The 5′ UTR to bp −516 was amplified (5′-GCTAGCGTGGACCCCCATTATCCTTCGC-3′ and 5′-ACTAGTCACTCGT CGAATGGTTGCGTCTG-3′), and 5′ UTR to bp -2,057 was amplified (5′-GCTAGCGTGGACCCCCATTATCCTTCGC-3′ and 5′-ACTAGTGA AATCGCGATCAACGCGACAG-3′). The 3′ UTR (920 bp) was amplified (5′-AGTACTTGCACCACCACCACCACCACTAATTTCCAATACTTTCGCCA AAAACGTTCC-3′ and 5′-GCGCACGTGGTTGAGAGCTTGACCCGCATG-CA-3′). Finally, the 5′ UTR segments (ScaI/SpeI) were fused into the CPSII minigene (SpeI), and subsequently the 3′ UTR (ScaI/PmlI) was fused into the above plasmid(s) (ScaI/PmlI).

2.2. Site-directed mutagenesis

Mutations were first introduced into either the GATase or CPS domains using Stratagene′s PCR-based QuikChange® II XL Site-Directed Mutagenesis Kit. Products were Dpn I digested, transformed into XL-10 Gold Ultracomp cells, and subsequently transferred into the full CPSII complementation vector. Forward and reverse complementary primers containing the desired mutations were used to create the desired mutations. Plasmids with correct coding region and engineered CPSII minigene mutation(s) were verified by sequencing prior to transfection experiments.

2.3. Parasite culture and transfection

Tachyzoites of strain *cps1-1* were maintained in human foreskin fibroblasts (HFFs) with or without uracil supplementation (300 mM) (Fox and Bzik, 2002). Wild-type or CPSII minigene plasmids containing defined mutations were transfected (20 mg) into the *cps1-1* background and selections were performed without drug addition, in the absence of uracil, using previously described methods (Fox and Bzik, 2002). Briefly 1×10^7 freshly isolated tachyzoites of strain *cps1-1* were transfected in 0.4 ml electroporation buffer (Donald and Roos, 1993). Growth and complementation in the absence of uracil supplementation was scored as described below. Transfected *cps1-1* parasites growing in the absence of uracil were cloned by limiting dilution.

2.4. Determination of parasite growth rate in cps1-1 complementation assays

Following transfection of strain *cps1-1* with wild-type or mutant CPSII minigenes fresh monolayers of HFF cells were infected in 5 ml of infection medium with 25% of the transfected parasites. At 2 h post-transfection monolayers were washed twice with PBS to remove parasites that had not invaded the cells and fresh infection medium was returned to cultures. At 36 h post-transfection the growth rate was measured by scoring tachyzoites per vacuole by examination of randomly selected vacuoles using light microscopy. Vacuoles containing one parasite per vacuole were excluded from counting. A total of 50 vacuoles with two or more parasites were scored to determine a mean of parasites per vacuole in each transient transfection assay. The growth rate was then converted to a relative doubling time based on a 36-h growth period. Experiments were independently repeated at least three times. A Student's *t*-test was used to calculate the SEM.

2.5. Determination of transient complementation efficiency

Following transfection of strain *cps1-1* with wild-type or mutant CPSII minigenes, fresh monolayers of HFF cells were infected in 5 ml of infection medium with 25% of the transfected parasites. At 2 h post-transfection monolayers were washed twice with PBS to remove parasites that had not invaded the cells and fresh infection medium was returned to cultures. At 36 h post-transfection the transient transfection efficiency was measured at the same time as growth rate by scoring the number of vacuoles containing two or more parasites per light microscope "field" at a fixed objective during the scoring of 50 vacuoles as described above in Section 2.4. Vacuoles with only one parasite per vacuole were excluded from counting. The transient complementation efficiency is reported as the percentage of control vacuoles observed using the wild-type Pc 4 CPSII minigene. Experiments were independently