International Journal for Parasitology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Succinctus

The shikimate pathway enzyme that generates chorismate is not required for the development of *Plasmodium berghei* in the mammalian host nor the mosquito vector

Hadi Hasan Choudhary [a], Pratik Narain Srivastava [a], Subhash Singh [b], Kota Arun Kumar [c], Satish Mishra [a,*]

[a] Division of Parasitology, CSIR-Central Drug Research Institute, Lucknow 226031, India
[b] CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
[c] Department of Animal Biology, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India

ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form 25 October 2017
Accepted 27 October 2017
Available online xxxx

Keywords:
Plasmodium
Malaria
Sporozoites
Shikimate pathway
Chorismate synthase

ABSTRACT

In *Plasmodium*, the shikimate pathway is a potential target for malaria chemotherapy owing to its absence in the mammalian host. Chorismate, the end product of this pathway, serves as a precursor for aromatic amino acids, Para-aminobenzoic acid and ubiquinone, and is synthesised by Chorismate synthase (CS). Therefore, it follows that the *Cs* locus may be refractory to genetic manipulation. By utilising a conditional mutagenesis system of yeast Flp/FRT, we demonstrate an unexpectedly dispensable role of CS in *Plasmodium*. Our studies reiterate the need to establish an obligate reliance on *Plasmodium* metabolic enzymes through genetic approaches before their selection as drug targets.

Malaria is a mosquito-borne infectious disease caused by a protozoan parasite which belongs to the genus *Plasmodium*. In 2015 alone, there were 212 million cases and 429,000 deaths due to malaria worldwide (W.H.O., 2016). The current challenges in curtailing malaria include a lack of effective vaccines and drugs that can target multiple life cycle stages. In the recent past, metabolic pathways unique to *Plasmodium* have been sought for the development of new antimalarial drugs as they are absent in humans. The shikimate pathway is one such example that uniquely operates in bacteria, fungi, plants and apicomplexan parasites (Roberts et al., 1998; McConkey, 1999). This pathway has seven steps that finally lead to the formation of chorismate from 5-enolpyruvylshikimate 3-phosphate (EPSP). Chorismate produced as the end product of this pathway serves as a substrate for the synthesis of aromatic amino acids, Para-aminobenzoic acid (PABA) and ubiquinones (Stenmark et al., 1974; Bentley, 1990; Herrmann and Weaver, 1999; Roberts et al., 2002). These aromatic compounds are essential for some of the most important biological processes of the parasite. For example, PABA is a precursor for folates that are required for nucleotide synthesis and the methylation cycle (Rebeille et al., 2006; Blancquaert et al., 2010; Ravanel et al., 2011). Ubiquinones are components of the electron transport chain, and in *Plasmodium* it is synthesised in two steps – the isoprene side chain synthesis occurs via a terpenoid pathway, and benzoquinone synthesis occurs via a shikimate pathway (Ginsburg, 2016).

The shikimate pathway enzymes can be potential targets for antimalarial drugs as exemplified by the potency of glyphosate, a herbicide that inhibits the activity of EPSP synthase, resulting in a significant reduction in *P. falciparum* growth in vitro (Kishore and Shah, 1988). The importance of this pathway was further reiterated by demonstrating only a partial inhibition of *P. falciparum* growth (nearly 30%) following depletion of PABA, folates and aromatic amino acids in medium (McConkey, 1999). These studies imply the reliance of the parasite on the de novo pathway to meet its aromatic metabolite requirements. Further, fluorinated analogues of shikimate also inhibited *P. falciparum* growth in vitro, that was reversed by addition of PABA (McConkey, 1999), implicating that this pathway supplies folates for the parasite. Paradoxically, however, glyphosate treatment of mice infected with *Toxoplasma*, a related apicomplexan parasite, does not suppress the infectivity. This points to the remarkable adaptability of the parasite to switch to the salvage source to procure the aromatic substrates when the de novo pathways are inhibited (Roberts et al., 1998). Thus while the dependence of the parasite on the shikimate pathway remains debatable, to our knowledge there have been no experimental genetic approaches used to deplete the enzymes of this pathway. One likely reason for this may be

* Corresponding author.
E-mail address: satish.mishra@cdri.res.in (S. Mishra).

https://doi.org/10.1016/j.ijpara.2017.10.004
0020-7519/

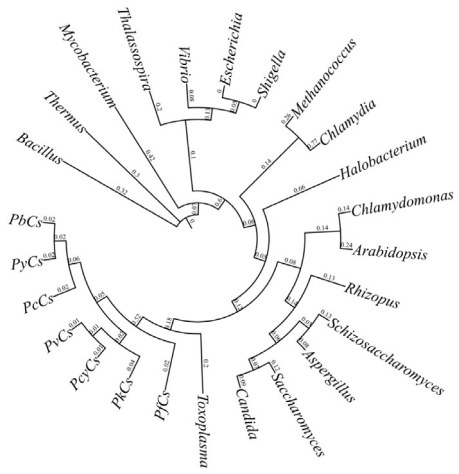
the assumption that with chorismate being highly indispensable for the parasite, such approaches may yield lethal phenotypes.

To address the importance of the shikimate pathway in *Plasmodium*, we resorted to conditional depletion of Chorismate synthase (*Cs*) (PlasmoDB ID: PBANKA_1121900) in mosquito stages of the parasite and analysed its role, if any, in the mammalian host. CS was strategically chosen as it was the immediate and only enzyme of this pathway required for chorismate synthesis. CS carries out 1, 4 trans elimination of the phosphate group of EPSP that results in the formation of chorismate, utilising Flavin mononucleotide (FMN) as a cofactor. (Bornemann et al., 1995; Macheroux et al., 1996). In *P. falciparum*, CS localises to the cytoplasm (Fitzpatrick et al., 2001) and interestingly silencing its expression by double-stranded RNA (dsRNA) resulted in a significant decrease in parasite growth (McRobert and McConkey, 2002). CS expression was also detected in the proteomic analyses of *P. falciparum* sporozoites (Lindner et al., 2013), likely implicating the need for chorismate and its products in establishing infection of the mammalian host.

We initiated characterization of CS by in silico analysis. To understand the evolutionary proximity of *Plasmodium* CS with other species, a phylogenetic tree was constructed using the PhyML module of the phylogeny.fr web server and visualised using the EMBL Interactive Tree Of Life (iTOL) service (Letunic and Bork, 2016). The tree shows a high degree of conservation among apicomplexans with the group containing fungi, *Arabidopsis* and *Chlamydomonas* being the closest neighbour (Fig. 1A). Sequence analysis of homologues using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo; version 1.2.4) revealed that CS proteins are conserved in all malaria parasites (Fig. 1B). To predict involvement of CS in possible biochemical pathways, the amino acid sequence of *P. falciparum* CS was submitted to the STRING interaction database (https://string-db.org). The STRING interaction diagram of *P. falciparum* CS shows that the enzyme contributes directly or indirectly to various biochemical pathways, emphasising its importance in parasite metabolism (Fig. 1C and Table 1). To further emphasize the importance of CS in the parasite life cycle, the *Plasmodium berghei* phenotypes of STRING-associated genes were extracted from *Plasmo*GEM (http://plasmogem.sanger.ac.uk/phenotypes). Among these genes, PlasmoDB IDs PBANKA_1032300 (PF3D7_1410200) and PBANKA_0823300 (PF3D7_0922400) were shown to be essential for parasite viability with known experimental association with CS, whereas PBANKA_1211000 (PF3D7_1012600) mutants demonstrated a significantly slow growth phenotype with a predicted association with *P. falciparum* CS (Bushell et al., 2017).

For genetic validation of CS, we used *Plasmodium berghei* ANKA strain, a rodent strain that is readily propagated in mice. All animal experiments performed in this study were approved by the Institutional Animal Ethics Committee at Council of Scientific and Industrial Research-Central Drug Research Institute, India (approval no: IAEC/2013/83). The conditional depletion of CS in mosquito stages was achieved by using a yeast-based Flp/FRT system routinely employed for functional investigation of genes that are essential in blood stage parasites (Combe et al., 2009). To generate conditional targeting construct, fragments 1, 2 and 3 (F1, F2 and F3) were amplified using primers 1087–1088, 1089–1090 and 1091–1092 respectively (primer sequences are provided in Supplementary Table S1). The fragments were cloned into the p3′trap-hDHFR-flirte2-puc18 vector using *Sph*I-*Not*I for F1, *Xho*I for F2 and *Pst*I-*Kpn*I for F3 (Fig. 2A). Fragment 2 consisted of a CS expression cassette flanked by Flp recognition target sequences (FRT sites). Following correct assembly of all fragments, the targeting cassette was separated from the vector backbone by digestion with *Sph*I/*Kpn*I and transfected into schizont stages of the *P. berghei* TRAP/FlpL parent line using the protocol as described earlier (Janse et al., 2006). This line expresses FlpL recombinase under the control of the TRAP promoter, active in the mosquito oocyst stage, and selectively excises the DNA sequence flanked by FRT sites (Combe et al., 2009; S. Mishra, K.A. Kumar and P. Sinnis, unpublished data). Successful integration of the targeting construct at the correct locus was confirmed by diagnostic PCR (Fig. 2B). Presence of the FRT site was confirmed by sequencing the amplified product using primers 1093–1095. Pyrimethamine drug-resistant parasites were single cloned by limiting dilution. To obtain a conditionally silenced mutant of *Cs*, two different clonal lines were propagated independently in two groups of mice, and female *Anopheles* mosquitoes were allowed to feed on the infected mice.

A



B

	*Pf*CS	*Pb*CS	*Pk*CS	*Pch*CS	*Pv*CS	*Pcy*CS	*Py*CS
*Pf*CS	100	74.9	72.8	74.9	70.9	58.6	71.7
*Pb*CS		100	73.9	87.1	67.8	61.0	91.9
*Pk*CS			100	73.6	83.5	71.9	72.7
*Pch*CS				100	70.4	58.5	84.8
*Pv*CS					100	68.7	65.9
*Pcy*CS						100	59.2
*Py*CS							100

C

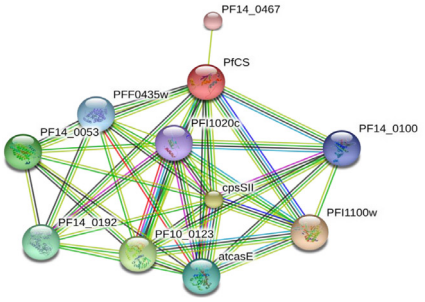


Fig. 1. Amino acid sequence analysis of chorismate synthase (CS). (A) Phylogenetic tree of chorismate synthase. The PlasmoDB IDs of different *Plasmodium* CS orthologues are- *Plasmodium falciparum* (*Pf*CS: PF3D7_0623000), *Plasmodium berghei* (*Pb*CS: PBANKA_1121900), *Plasmodium knowlesi* (*Pk*CS: PKNH_1127200), *Plasmodium chabaudi* (*Pc*CS: PCHAS_1121400), *Plasmodium vivax* (*Pv*CS: PVX_114265), *Plasmodium cynomolgi* (*Pcy*CS: PCYB_113480) and *Plasmodium yeolii* (*Py*CS: PY04071). (B) The table shows the CS amino acid sequence similarity matrix amongst *Plasmodium* orthologues. The Clustal Omega program was used to compare identities. (C) STRING interaction diagram of *Pf*CS, showing its known interactions with a variety of enzymes, further emphasises its possible importance in the cell. On the basis of various criteria used by the STRING database, *Pf*CS can be associated with Gamete egress and sporozoite traversal protein, putative (PF3D7_1449000), Ornithine aminotransferase (PF3D7_0608800), Inosine-5′-monophosphate dehydrogenase (PF3D7_0920800), Cytidine triphosphate synthetase (PF3D7_1410200), Ribonucleoside-diphosphate reductase small chain, putative (PF3D7_1405600), Carbamoyl phosphate synthetase (PF3D7_1308200), Para-aminobenzoic acid synthetase (PF3D7_0922400), Glutathione reductase (PF3D7_1419800), Guanosine monophosphate synthase (PF3D7_1012600) and Aspartate carbamoyltransferase (PF3D7_1344800).

Please cite this article in press as: Choudhary, H.H., et al. The shikimate pathway enzyme that generates chorismate is not required for the development of *Plasmodium berghei* in the mammalian host nor the mosquito vector. Int. J. Parasitol. (2018), https://doi.org/10.1016/j.ijpara.2017.10.004