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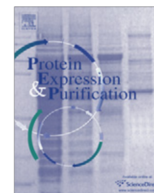

Overexpression, purification and assessment of cyclosporin binding of a family of cyclophilins and cyclophilin-like proteins of the human malarial parasite *Plasmodium falciparum*

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ABSTRACT

Malaria represents a global health, economic and social burden of enormous magnitude. Chemotherapy is at the moment a largely effective weapon against the disease, but the appearance of drug-resistant parasites is reducing the effectiveness of most drugs. Finding new drug-target candidates is one approach to the development of new drugs. The family of cyclophilins may represent a group of potential targets. They are involved in protein folding and regulation due to their peptidyl-prolyl *cis–trans* isomerase and/or chaperone activities. They also mediate the action of the immunosuppressive drug cyclosporin A, which additionally has strong antimalarial activity.

In the genome database of the most lethal human malarial parasite *Plasmodium falciparum*, 11 genes apparently encoding cyclophilin or cyclophilin-like proteins were found, but most of these have not yet been characterized. Previously a pET vector conferring a C-terminal His_6 tag was used for recombinant expression and purification of one member of the *P. falciparum* cyclophilin family in *Escherichia coli*. The approach here was to use an identical method to produce all of the other members of this family and thereby allow the most consistent functional comparisons. We were successful in generating all but three of the family, plus a single amino-acid mutant, in the same recombinant form as either full-length proteins or isolated cyclophilin-like domains. The recombinant proteins were assessed by thermal melt assay for correct folding and cyclosporin A binding.

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Introduction

According to the latest report of the World Health Organization [1], about 3.3 billion people are at risk of malaria, leading to about 250 million cases a year and nearly one million deaths. In Africa alone it contributes to the death of about 10,000 pregnant women and 700,000 children each year. By far the most significant species is *Plasmodium falciparum*, which causes severe infections and death, and enjoys widespread geographic distribution [2]. Chemotherapy plays a major role in malaria control but the antimalarial drug armory must constantly be renewed because drugs are continually falling prey to resistance [3]. One approach to new drug development lies in the identification of novel drug targets in the parasite, guided by analysis of genome sequence data [4]. Due to the almost overwhelming obstacles to purifying all but the most abundant *P. falciparum* proteins directly from the parasites, potential target proteins are typically produced in recombinant form for structural analysis and measurement of binding and/or inhibition of activity by small molecules [5].

P. falciparum is well known for having genes that can be particularly resistant to heterologous expression in bacterial and yeast hosts. A variety of factors are thought to account for this: the genome is 80% AT and has a codon bias well removed from that of *Escherichia coli*; glycosylation patterns unique to the parasite are utilized [6]; and *P. falciparum* proteins are generally larger than homologs in other species, for example as much as 50% larger on average than homologs in *Saccharomyces cerevisiae* [7]. In addition, it is not uncommon for *P. falciparum* genes to contain cryptic start sites for *E. coli* that might produce mRNAs with altered protein-coding sequences or translation efficiencies resulting in multiple, truncated products when overexpressed in these bacteria [8]. Despite the difficulties, there are many examples of *P. falciparum* proteins expressed in *E. coli* and with the simplicity and speed of the T7 expression vector system, *E. coli* is the host of choice for most of the groups studying structural genomics [9]. The vector used often adds an oligohistidine affinity tag [10] that is relatively small,

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allows purification by a relatively simple protocol using immobilized metal affinity chromatography (IMAC[1]) [11], and rarely affects the functional characteristics or solubility of the tagged protein [12].

The immunosuppressive drug cyclosporin A (CsA) was shown in the early 1980s to have anti-malarial activity in cultured blood-stage parasites and in vivo, and even to reduce the severity of malaria in experimental infections of owl monkeys [13–15]. The immunosuppressive effects of cyclosporins have limited their development as antimicrobial agents, but the appearance of non-immunosuppressive derivatives of CsA with pronounced antimalarial activity emphasized the importance of further investigating the action of these drugs upon the parasite [14–16]. In humans, by inhibiting signaling through the phosphatase calcineurin to a combination of transcription factors, CsA suppresses the transcriptional activation of the interleukin-2 gene [17]. Of these transcription factors, the nuclear factor of activated T-cells appears to be the most sensitive to the drug and is the only pathway for which the molecular mechanism of CsA-immunosuppression is well characterized. CsA exerts its action as part of a complex with its receptor, a member of the family of proteins called cyclophilins [17,18]. The mechanism(s) of antimalarial action of CsA and other cyclosporins is/are unknown but two major cyclosporin receptors have been identified, namely the parasite cyclophilins PfCYP19A and PfCYP19B [19,20]. The precise role of these two proteins in the antimalarial action of these compounds is not yet clear [20]. Moreover, 10 other cyclophilin-related gene sequences are present in *P. falciparum* genomes, and some of them are expressed in blood-stage parasites, but their contribution if any to cyclosporin action is unknown. The chemically unrelated immunosuppressant FK506, which acts analogously to CsA following binding to its FK506-binding protein (FKBP) receptor, also has antimalarial activity [16].

Regardless of their origin, the structural conservation of cyclophilins throughout evolution and the presence of peptidyl-prolyl *cis–trans* isomerase (PPIase) activity in most members, and molecular-chaperone activity in some, underline their importance and suggest that cyclophilins may play an important role in malarial parasite development [18,20]. So far, two recombinant *P. falciparum* cyclophilins PfCYP19B and PfCYP19A [21,22] (see Ref. [20] for nomenclature) have been successfully purified and biochemically characterized. Functionally active PfCYP19B (minus its secretory signal peptide) was produced in milligram quantities in *E. coli* using the pET22b+ vector, which generated a protein with a C-terminal His_6 tag [21]. Similar approaches have been used in high-throughput expression of various *P. falciparum* genes [9]. Since our intention was to compare the functional properties and cyclosporin-binding capacity of all of the cyclophilin/cyclophilin-like family members, we wished to obtain recombinant proteins under conditions as close to identical as possible. We therefore adopted the pET system with a C-terminal His_6-tag for 'medium-throughput' production of this family of proteins.

Materials and methods

Materials

All chemicals were obtained from Sigma–Aldrich (Dublin, Ireland) unless otherwise stated.

Routine culture of P. falciparum

The *P. falciparum* 3D7 strain was maintained in continuous culture in human erythrocytes according to the method of Trager and Jensen [23]. Parasites were cultured routinely in culture medium [RPMI 1640 medium supplemented with 25 mM HEPES, 0.01% (w/v) gentamicin, 0.18% (w/v) sodium bicarbonate, hypoxanthine (50 μg/ml), 10% albumax® (a serum substitute: Invitrogen, Dun Laoghaire, Ireland)] and washed erythrocytes [at a hematocrit of 2.5% or 5% (v/v)]. Parasites were maintained in Petri dishes at 37 °C in a candle jar. Culture medium was replaced depending on the parasitaemia, which was monitored by microscopic examination of Giemsa-stained smears.

Harvesting of parasites

Free parasites were released from infected erythrocytes according to the saponin method of Zuckerman et al. [24]. Freed parasites were sedimented by centrifugation at $975 \times g$ at 4 °C for 15 min and washed twice with cold saline sodium citrate buffer. Pellets were either used immediately or resuspended in freezing solution [10% (v/v) glycerol, 2 mM phenylmethylsulphonyl fluoride, pepstatin A (1 μg/ml), leupeptin (20 μg/ml), prepared in phosphate-buffered saline] for storage at −80 °C.

Cloning of P. falciparum cyclophilin and cyclophilin-like genes: amplification by PCR

Of the cyclophilin or cyclophilin-like open-reading frames discussed below in the 'Results' section, six were found not to have any introns, thus genomic DNA could be used as template. Primers displayed in Table 1 were used to amplify their coding sequences, with *Nde*I and *Xho*I sites at the 5′ and 3′ ends, respectively, to facilitate subsequent cloning into the pET22b+ expression vector. Note that, where indicated, due to the presence of other domains within the gene and difficulties in amplifying large AT-rich regions, only the cyclophilin-like domain (CLD) was amplified. DNA and RNA were isolated from harvested parasites as described elsewhere [25].

PCR was performed using ~1 μg *P. falciparum* 3D7 genomic DNA, 0.5 μM primers, 2 U *Pfu* turbo® DNA polymerase (Stratagene, La Jolla, CA, USA) and 0.5 mM each of dATP, dTTP, dGTP and dCTP (Stratagene) in a TC-3000 thermocycler (Techne, Staffordshire, UK) (95 °C for 5 min; followed by 40 cycles of 95 °C for 30 s, 45 °C for 1 min, 72 °C for 3 min; and a last step of 72 °C for 10 min).

Amplification by reverse transcriptase PCR (RT-PCR)

The other cyclophilin/cyclophilin-like genes showed at least one predicted intron in their sequences, although one of these (*pfcyp19B*) had already been cloned [21]. Therefore a two-stage RT-PCR was performed on total RNA isolated from *P. falciparum* 3D7. Firstly, cDNA synthesis was performed on ~2 μg RNA in the presence of a specific primer, pre-incubated at 70 °C for 10 min. Thus, *Nde*I (or *Nco*I) and *Xho*I sites were incorporated. Then 0.5 μM of each dNTP, 10 mM dithiothreitol (New England Biolabs, Ipswich, MA, USA) and 30 U of avian myeloblastosis virus reverse transcriptase (USB Products, Stauffen, Germany) were added. The samples were incubated at 42 °C for 30–60 min, followed by 10 min at 70 °C to inactivate the enzyme. Amplification of cDNA was performed as described before, except with the template being cDNA rather than genomic DNA. In all cases PCR products were purified using the quick protocol of a wizard SV® gel and PCR clean-up system (Promega, Wisconsin, USA).

Cloning of putative P. falciparum cyclophilin genes into pET22b+ or pET21d+

pET22b+ and pET21d+ (Novagen, Darmstadt, Germany) were isolated from *E. coli* XL-1 Blue cells (Stratagene) using a wizard *plus*

[1] *Abbreviations used:* CLD, cyclophilin-like domain; CsA, cyclosporin A; FKBP, FK506-binding protein; IMAC, immobilized metal affinity chromatography; PPIase, peptidyl-prolyl *cis–trans* isomerase; RT, reverse transcriptase.

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