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Characterisation of a sexual stage-specific gene encoding ORC1 homologue in the human malaria parasite \textit{Plasmodium falciparum}\(^\text{\textsuperscript{\textregistered}}\)

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Abstract

The origin recognition complex (ORC) is a multisubunit protein composed of six polypeptides that binds to replication origins and is essential for the initiation of chromosomal DNA replication. Using the Vectorette technique, we have isolated a novel gene encoding an ORC1-like protein from the human malaria parasite \textit{Plasmodium falciparum}. The gene has no introns and encodes a protein (PfORC1) of 1189 amino acid residues with a predicted molecular mass of 139 kDa. PfORC1 contains all conserved sequences in the ORC1/Cdc6/Cdc18 family and displays the highest homology to the \textit{Schizosaccharomyces pombe} ORC1. However, PfORC1 possesses an extensive N-terminal segment with several interesting features including multiple potential phosphorylation sites, a large proportion of charged amino acids, four copies of a heptamer repeat, two nuclear localisation signals, and a leucine zipper motif. Southern blot analyses show that the \textit{Pforc1} gene is present as a single copy per haploid genome and is located on chromosome 12. A 5600 nucleotide transcript of this gene is expressed predominantly in the sexual erythrocytic stage, indicating that PfORC1 may be involved in gametogenesis during which DNA is quickly replicated.

Keywords: Malaria; Origin recognition complex; DNA replication initiation; \textit{Plasmodium falciparum}; Gametocyte

1. Introduction

Malaria kills between 1 million and 3 million humans, mostly children, every year, making it the leading cause of death from an infectious disease [1]. The lack of an effective vaccine and the development of \textit{Plasmodium} resistance to many existing antimalarial drugs have aggravated the situation. It is therefore imperative that our understanding of the fundamental biology and biochemical processes at different stages of the parasite be improved, to facilitate the identification of new targets for the development of novel drugs and vaccines. DNA replication represents such a key process of the parasite. There are at least five distinct points in the parasite life cycle when DNA replication occurs [2], two of which take place in the human host, i.e. in the hepatocytes and in the erythrocytes, and the remainder occur in the mos-
quito vector. The selective blocking of DNA synthesis in the parasite should inhibit both the disease itself and the parasite transmission. DNA replication proceeds in two continuous phases: initiation and elongation. To date, only four components involved in DNA replication elongation have been identified and isolated from \textit{Plasmodium falciparum} [2]. These include proliferating cell nuclear antigen (PCNA) [3], primase [4], DNA polymerase \(\alpha [5-7]\) and DNA polymerase \(\delta [8,9]\). However, there is no information concerning the DNA replication initiation in the parasite. We are interested in DNA replication in \textit{P. falciparum}, particularly at the initiation stage. The first step toward this goal has been to identify the essential components of the initiation machinery from the parasite. Recently, we have isolated an MCM4 protein and characterised of a novel gene encoding a \textit{Plasmodium falcipa}-

### 2. Materials and methods

#### 2.1. Parasite and parasitic materials

\textit{P. falciparum} clones T996 and 3D7A were used in this study. Parasite DNA and total RNA were extracted from cultures of \textit{P. falciparum} 3D7A as described previously [11]. The first-strand cDNA was generated from 1 \(\mu\)g of total RNA using the random hexamer primer in the rapid-amplification-of-\(5\)'-cDNA-ends system of synthesis (Gibco) with DNase I [12].

#### 2.2. Oligonucleotides


#### 2.3. Construction and screening of Vectorette libraries

Vectorette libraries were constructed from \textit{P. falciparum} 3D7A genomic DNA as described previously [11]. With a specific primer and a universal Vectorette primer, PCR was used to screen Vectorette libraries [11]. PCR products were cloned into the pGEM-T vector (Promega) and sequenced using an ABI PRISM (model 377) automatic sequencing facility in this department.

#### 2.4. Southern and Northern blot analyses

Approximately 4 \(\mu\)g of genomic DNA (clone 3D7A) digested with a number of restriction enzymes was used for Southern blotting and approximately 10 \(\mu\)g of total RNA extracted from both asexual and sexual erythrocytic stages of \textit{P. falciparum} 3D7A was employed for Northern blotting [11]. The Hybond N nylon membrane blots containing DNA or RNA were probed with a \(32\)P-labelled \(\text{ORM} \)-\(\text{OR}B \) fragment as described previously [11]. Filters were washed at 56°C in 1× SSC/0.1% SDS for 40 min and then in 0.5× SSC/0.1% SDS for 30 min and autoradiographed at \(-80^\circ\)C.

#### 2.5. Pulse-field gel electrophoresis

Preparation of the agarose blocks containing chromosomal DNA, gel electrophoresis using a
Fig. 1. A schematic representation of a partial restriction map of the \textit{Pforc1} gene and the overlapping fragments used to determine its nucleotide sequence. The open box represents the coding region of the \textit{Pforc1} gene. The fragments of 0451C3 and 1424C3 were obtained from the cDNA tag database. The fragments of OR2-BclI, OR3-Rsal, OR4-HincII, OR7-HincII, OR7-TaqI, OR8-TaqI, OR10-Rsal, OR10-AluI, OR11-TaqI, OR12-TaqI, OR13-Rsal and OR15-Sau3A were derived from the Vectorette PCRs. OR\textsubscript{M}–OR\textsubscript{E} and OR\textsubscript{M}–OR\textsubscript{E} were amplified from genomic DNA.

Bio-Rad CHEF DRII system, and treatment of the blotted membrane were carried out as described previously [12]. The blot was hybridised with the OR\textsubscript{M}–OR\textsubscript{E} probe and processed further as described above.

3. Results

3.1. Identification and isolation of the \textit{Pforc1} gene

In the \textit{P. falciparum} tag database there were two expressed sequence tags (0451C3 and 1424C3) that encode two protein fragments both with a high sequence homology to the \textit{Xenopus} origin recognition complex subunit 1 (ORC1). Nucleotide sequence analysis revealed that the A+T content and codon usage of both tag sequences are typical of the coding region of \textit{P. falciparum} genes [13]. To isolate a full-length of the gene, two specific primers, OR2 (to obtain further sequence in the 5' direction) and OR3 (to obtain further sequence in the 3' direction), were constructed on the basis of the 0451C3 sequence and used in PCR to screen Vectorette libraries [11]. Two fragments (OR2-BclI, and OR3-Rsal) were obtained and sequenced (Fig. 1). The OR2-BclI fragment, as predicted, covered the whole 1424C3 tag sequence. The sequence data of OR2-BclI and OR3-Rsal permitted synthesis of OR4 and OR11 respectively and subsequent amplification of the OR4-HincII and OR11-TaqI fragments from the Vectorette libraries. The known sequences from OR4-HincII and OR11-TaqI made it possible to construct the OR8 and OR13 primers that gave rise to the OR8-TaqI and OR13-Rsal fragments respectively. Based on these two sequences, the OR10 and OR15 primers were synthesised and used to screen Vectorette libraries further. Two overlapping PCR products (OR10-Rsal and OR10-AluI) were amplified with OR10, whereas only one fragment (OR15-Sau3A) was obtained with OR15. The sequence data of OR10-AluI allowed construction of the OR12 primer that consequently generated the OR12-TaqI fragment. Based on OR12-TaqI, the OR7 primer was synthesised and used to generate OR7-HincII and OR7-TaqI. As expected, OR7-HincII contains all sequence information of OR7-TaqI. In order to confirm the sequence obtained from the overlapping fragments, several independent PCR fragments (such as OR\textsubscript{M}–OR\textsubscript{E} and OR\textsubscript{M}–OR\textsubscript{E}) were amplified from the parasite.
3D7A) genomic DNA and sequenced in both strands (Fig. 1).

3.2. Characteristics of the nucleotide sequence of the Pforc1 gene

The sequence derived from overlapping PCR fragments consists of 5113 bp and contains an open reading frame starting with an ATG codon at nucleotide 722 and terminating with a TAG codon at nucleotide 4289. In both flanking regions (upstream of the putative start codon and downstream of the putative stop codon), there are multiple in-frame stop codons for all three possible reading frames. The sequence and codon usage in the coding region are typical for a |

3.3. Characteristics of the predicted PfORC1 protein

The open reading frame encodes a protein of 1189 amino acids (Fig. 2) with a predicted molecular mass of approximately 138.7 kDa and an isoelectric point 9.525. Database searches revealed that the amino acid sequence of the predicted protein shares 48–61% similarity and 27–40% identity with proteins in the ORC1 family across the conserved region (residue positions 741–1088) and displays the highest homology (40% identity, 59% similarity) to the Schizosaccharomyces pombe ORC1 [16,17]. Accordingly, we designated it as PfORC1. PfORC1 is composed of two distinct domains: (1) a variable N-terminal domain, and (2) a highly conserved C-terminal domain (Fig. 2). The N-terminal domain consists of 783 amino acid residues and possesses several interesting features. First, it is rich in serine/threonine and tyrosine (20%), forming a number of potential phosphorylation sites for a range of known protein kinases [18]. Second, a large proportion (30%) of charged amino acids (K, R, E and D) exists in the N-terminal segment. Third, there are four copies of a heptamer ISSSLT(S)N repeats positioned between 133 and 161. Fourth, the N-terminal segment contains two putative nuclear localisation signals [19]. Fifth, the N-terminal domain possesses a putative leucine zipper motif (L-X<sub>5</sub>-L-X<sub>8</sub>-L-X<sub>5</sub>-L) at residue positions 137–158 [20]. Finally, the N-terminal segment is the largest extension in the ORC1 family yet described, representing a unique targeting point for the development of novel anti-malarial drugs. The C-terminal domain is composed of 406 amino acid residues and contains all conserved sequences in the ORC1 family including the Walker A and B motifs of the classical purine nucleotide-binding sites [21]. The Walker A motif interacts with the triphosphate moiety and is essential for ATP binding whereas the Walker B motif coordinates the Mg<sup>2+</sup> ions via a water molecule.

3.4. Structural organisation of the Pforc1 gene

To determine the copy number of the Pforc1 gene in the P. falciparum genome, 3D7A genomic DNA was digested with various restriction enzymes and analysed by Southern blotting. Hybridisation of the OR<sub>M</sub>-OR<sub>E</sub> (see Fig. 1) probe revealed a single band in digests with AccI, BamHI, EcoRI or HincII, for which there is no restriction site in the OR<sub>M</sub>-OR<sub>E</sub> fragment, two bands in EcoRV digests, for which only one restriction site exists in the probe, and three bands on digestion with BclI, for which two restriction sites exist in the fragment (data not shown). These results suggest strongly that PfORC1 is encoded by a single copy gene in the parasite genome. To
investigate the chromosome location of the \textit{Pforc1} gene, \textit{P. falciparum} (3D7A and T996) chromosomes were resolved on the CHEF gel system, blotted onto a nylon membrane and hybridised with the OR\textsubscript{M}–OR\textsubscript{V} probe. A single band was detected corresponding to chromosome 12 on two independent blots (Fig. 3c and data not shown). The result was further confirmed by probing these blots with control probes derived from the \textit{Pfpcna} gene (Fig. 3d) and the \textit{Pfmap2} gene (Fig. 3b), which are known to be located on chromosome 13 [3] and chromosome 11 (Li and Cox, unpublished data) [22], respectively. The result also supports the conclusion that \textit{Pforc1} is present as a single-copy gene per haploid genome.

3.5. Stage-specific expression of the \textit{Pforc1} gene

To obtain some information on how \textit{Pforc1} mRNA levels are regulated during parasite development and differentiation, a Northern blot containing equal quantities of total RNA prepared
Fig. 3. Chromosomal localisation of the Pf orc1 gene. Parasite chromosomes from P. falciparum 3D7A and T996 were separated by pulse-field gel electrophoresis, stained with ethidium bromide, blotted onto a nylon membrane and hybridised with radiolabeled probes. Based on the yeast chromosome markers and hybridisation of several P. falciparum chromosome marker genes, the positions of chromosome 10, 11, 12, 13 and 14 were identified on the ethidium bromide-stained gel (a), Pfmap2 (b), Pf orc1 (c) and Pfpcna (d) hybridised to chromosome 11, 12 and 13, respectively.

from cultures enriched in stage III to stage V gametocytes and from mixed asexual erythrocytic stages was probed with the ORSm–ORSe fragment (see Fig. 1). A single transcript of approximately 5600 nucleotides in size was detected predominantly in the lane containing the sexual stage RNA, migrating behind the 28S ribosomal RNA band (Fig. 4a). Assuming a 100–200 residue poly(A) tail, the result implies that the mature transcript of Pf orc1 may contain approximately 1800 nucleotides of 5' and 3' untranslated sequences. As internal controls for hybridisation to the asexual and sexual stage mRNA, the same blot was hybridised with Pf lammer, a sexual stage-specific gene [23], and Pf pkac, an asexual stage-specific gene [24] (Fig. 4b,c).

3.6. Pf ORC1 is a member of the ORC1/Cdc6/Cdc18 family

Sequence analysis by database searches has also revealed that Pf ORC1 has significant sequence homology to the Cdc6/Cdc18 proteins (25–36% identity, 43–55% similarity) with the highest homology to human Cdc6 (36% identity, 55% similarity) in the N-side of the C-terminal domain from amino acids 784–1018 (Fig. 5). Within this conserved region, there are six blocks of strong similarity, defining Pf ORC1 as a member of the ORC1/Cdc6/Cdc18 family. Blocks 1 and 3 overlap the match to the Walker A (P-loop) and B (A-loop) motifs as described above, suggesting that the region of similarity may be responsible for NTP binding and hydrolysis. Therefore, this conserved region is referred to as the CDC-nucleoside triphosphate-binding (CDC-NTP) domain [25]. Comparison of the amino acid sequences in the CDC-NTP domain has made it possible to draw a phylogenetic tree (Fig. 6). Four vertebrate proteins, hamster ORC1, mouse ORC1, human ORC1 and frog ORC1, are closely related to each other, and so are four yeast proteins. Pf ORC1 seems to be more closely related to S. pombe ORC1, rice
ORC1 and Arabidopsis ORC1 than to the vertebrate proteins.

4. Discussion

The initiation of DNA replication in eukaryotes is tightly regulated such that all genomic DNA is replicated precisely once per cell cycle from a number of discrete replication origins [26]. ORC is a key protein for the initiation of chromosomal DNA replication. Initially identified in Saccharomyces cerevisiae, ORC is a multisubunit protein composed of six polypeptides (ORC1-ORC6) that binds to yeast replication origins in vivo and in vitro [26,27]. Homologues of individual subunits of the S. cerevisiae ORC have been identified in S. pombe, Drosophila melanogaster, Arabidopsis thaliana, Xenopus laevis, and human cells [16,25,28–33]. In this study, we have isolated and characterised a novel gene from P. falciparum encoding PfORC1, the largest subunit of ORC. The identification of PfORC1 may provide information that can help to identify replication origin sequences of the human malaria parasite.

Analysis of the amino acid sequences revealed that PfORC1 consists of two distinct domains. In the N-terminal segment, there are a number of potential phosphorylation sites for a range of known protein kinases including protein kinase C (PKC), casein kinase II (CKII), cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), cyclin-dependent kinases (Cdk) and tyrosine protein kinases, indicating that the activity of PfORC1 may be regulated by reversible phosphorylation of the N-terminal segment. Although the parasite PKC, CKII and tyrosine kinase have not yet been described, PKA [24], PKG [34] and Cdk [11,35] have already been identified in P. falciparum. It is worth noting that several sexual stage-specific protein phosphatases have also been demonstrated in P. falciparum [12,36] (Li et al., unpublished data). Indeed, it has been proved that in human Cdk2/cyclin A and Cdc2/cyclin B are capable of phosphorylating in vitro the N-terminal region of human ORC1 between residues 247 and 502 [37]. The higher ratio of charged amino acids occurs in the N-terminal domain of PfORC1, suggesting that this region may be involved in protein-protein interactions. It has been reported that a number of proteins are able to bind the N-terminal region of ORC1. These include the HP1 protein in Drosophila [38], the SIR1 protein in S. cerevisiae [39], and the histone acetyltransferase HBO1 and c-Myc in human [40,41]. The putative nuclear localisation signals exist in the N-terminal domain of PfORC1, suggesting that this region may be involved in protein-protein interactions. It has been reported that a number of proteins are able to bind the N-terminal region of ORC1. These include the HP1 protein in Drosophila [38], the SIR1 protein in S. cerevisiae [39], and the histone acetyltransferase HBO1 and c-Myc in human [40,41]. The putative nuclear localisation signals exist in the N-terminal domain of PfORC1, implying that PfORC1 may be a nuclear protein rather than a mitochondrial or plastid molecule. The leucine zipper motif in the N-terminal segment suggests that PfORC1 may have a DNA-binding property, consistent with the predicted function of PfORC1 in parasite DNA replication. In the C-terminal segment, there is the CDC-NTP domain. The CDC-NTP domain contains six blocks of strong similarity in the ORC1/Cdc6/Cdc18 family including the classical Walker A and B motifs, suggesting that PfORC1 may possess ATP binding.
Fig. 5. Sequence alignment of the ORC1 proteins with the Cdc6/Cdc18 proteins across the CDC-NTP region. The GenBank™/EMBL/DDJB database accession numbers are as follows: Kluyveromyces lactis KJ0RC1, P54784; Candida albicans CaORC1, Q74270; S. pombe SpORC1, P54789; Mus musculus MmORC1, Q9Z1N2; Cricetulus griseus CgORC1, O74270; Homo sapiens HsORC1, Q13415; Xenopus laevis XlORC1, U67190; Drosophila melanogaster DmORC1, O16810; Arabidopsis thaliana AtORC1, AC005496; and S. cerevisiae ScORC1, P09119. Sequences were aligned with the CLUSTAL W programme. The amino acid residues are numbered to the left of the sequence. Identical residues are indicated with asterisks and conservative changes indicated with dots. Blocks 1–6 are labelled on the top of sequence.
<table>
<thead>
<tr>
<th>Block 4</th>
<th>Block 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AtCdc6</strong> 268</td>
<td>LTTLPRLSRLIQLTVANANLDLARFLP-KLKSLSNCKF-LVVTFRAYSQKIDRLQKELVA</td>
</tr>
<tr>
<td><strong>ScCdc6</strong> 248</td>
<td>LAKLPVPSFLVIGMNASLMDKLDRFLS-RLLNLRGQLPQTIVQPYTAQMYEVITIVQKMS-</td>
</tr>
<tr>
<td><strong>HsCdc6</strong> 303</td>
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</tr>
<tr>
<td><strong>MmCdc6</strong> 332</td>
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<tr>
<td><strong>XlCdc6</strong> 295</td>
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<tr>
<td><strong>SpCdc18</strong> 305</td>
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</tr>
<tr>
<td><strong>KlCOC1</strong> 577</td>
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<tr>
<td><strong>ScCOC1</strong> 585</td>
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<tr>
<td><strong>CaCOC1</strong> 533</td>
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<tr>
<td><strong>SpCOC1</strong> 473</td>
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<tr>
<td><strong>McCOC1</strong> 618</td>
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<tr>
<td><strong>CgCOC1</strong> 628</td>
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<tr>
<td><strong>DmCOC1</strong> 703</td>
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**Fig. 5. (Continued).**
and hydrolysis activity. It has been demonstrated that *S. cerevisiae* ORC1 can bind and hydrolysis ATP, and the ORC1 ATP binding, but not the hydrolysis, is responsible for the ATP dependence of ORC DNA binding [42]. The relatedness of ORC1 and Cdc6/Cdc18 and their likely role in the early steps of DNA replication indicate that the conserved regions of these proteins may perform similar functions related to the initiation of DNA replication. It has been demonstrated that ORC1 interacts with Cdc6 in vitro and in vivo [43,44] and the interaction may help ORC in recruitment of Cdc6 to the replication origins, which then permits the recruitment of other essential initiation components such as minichromosome maintenance (MCM) proteins and Cdc45 [26].

Northern blot analysis revealed that a single transcript of approximately 5600 nucleotides is predominantly present in the sexual stages of the parasite, suggesting that PfORC1 is actively involved in sexual stage-specific events. It has been demonstrated that PfMCM4 is also specifically expressed in the sexual stages [10]. The quantity of DNA in mature gametocytes of *P. falciparum* has been shown to reach the diploid value [45], implying either complete genome duplication or selective gene amplification per haploid genome, consistent with results of electron microscopic studies [46]. Upon activation, the microgametocyte develops rapidly in the mosquito midgut (gametogenesis). Three successive rounds of genome replication are completed within 10 min, raising the DNA contents to octoploid values just before exflagellation, indicating that the genome duplication rate of the malaria parasite is extremely high, probably among the highest recorded. Assuming that the rate of replication fork movement in *Plasmodium* is similar to that in other eukaryotes, at least 1300 origins of replication would be needed to achieve this rate of replication [47]. The high level expression of PfORC1 in the sexual stage of *P. falciparum*, therefore, is consistent with its potential role in replication initiation during the sexual stage development, particularly during gametogenesis. Identification of other ORC subunits and other components including MCM, Cdc6/Cdc18 and Cdc45 homologues in the parasite will help afford new insight on the DNA replication initiation mechanism of the human malarial pathogen.

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