Multiple replication origins within the inverted repeat region of the Plasmodium falciparum apicoplast genome are differentially activated

Divya Singh, Ambrish Kumar, E.V.S. Raghu Ram, Saman Habib

Division of Molecular and Structural Biology, Central Drug Research Institute, P.O. Box 173, Chatter Manzil, Mahatma Gandhi Marg, Lucknow 226001, India

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Abstract

The 35 kb circular genome (plDNA) of the Plasmodium falciparum apicoplast replicates by the bidirectional ori/D-loop mechanism. PlDNA replication was previously shown to initiate within the inverted repeat (IR) region of the apicoplast genome [Williamson DH, Preiser PR, Moore PW, McCready S, Strath M, Wilson RJM (Iain). The plastid DNA of the malaria parasite Plasmodium falciparum is replicated by two mechanisms. Mol Microbiol 2002;45:533–42; Singh D, Chaubey S, Habib S. Replication of the Plasmodium falciparum apicoplast DNA initiates within the inverted repeat region. Mol Biochem Parasitol 2003;126:9–14.] and the presence of at least two ori within each segment of the IR was postulated. Using 5′ end-labelled nascent DNA as probe, we now demonstrate the utilization of several putative ori located within the IR for plDNA replication. Quantitation of signals obtained for different segments of the IR as well as determination of the number of molecules emanating from two ori regions by competitive PCR analysis indicated differential strengths of ori during plDNA replication prior to schizogony.

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1. Introduction

The plastid-like organelle (apicoplast) of Plasmodium falciparum has generated immense interest as a target for drugs against malaria [3,4]. The apicoplast is believed to be the site for type II fatty acid biosynthesis [5,6], the non-mevalonate pathway of isoprenoid biosynthesis [7,8], as well as synthesis of heme-intermediates within the parasite [9]. It contains a 35 kb circular genome (plDNA) that carries genes for large and small subunit RNAs as well as a minimal set of tRNA and ribosomal genes that are likely to participate in translation of protein-encoding genes on the circle [10].

The P. falciparum apicoplast exists as a branched structure in the late trophozoite-early schizont stages while multiple, discrete, slightly rod-shaped apicoplasts are visible at cytokinesis [11]. PlDNA replication starts in late trophozoites, a few hours before the onset of schizogony, and seems to coincide with total DNA synthesis in the parasite [12–14]. Each sporozoan cell of the parasite carries a single apicoplast with estimates of copy number of plDNA varying from 1 to 15 [15,16]. Replication of P. falciparum plDNA is inhibited by ciprofloxacin that targets DNA gyrase [1,17]. Additionally, ciprofloxacin and clindamycin block plDNA replication in the related apicomplexan Toxoplasma gondii and also inhibit parasite growth in culture [17].

The plDNA of P. falciparum and T. gondii share high sequence similarity but differ in their in vivo topologies [18]. The former has a circular topology while the latter exists primarily as a precise oligomeric series of linear tandem arrays of the basic 35 kb genome [12,18]. This difference in structure has important implications for the mode of plDNA replication. While T. gondii plDNA may replicate via the rolling circle mode [18], plDNA of P. falciparum is likely to follow
the bidirectional replication mode reported for most chloroplast genomes [19–22]. Reports by D.H. Williamson et al. [1] and our laboratory [2] have shown that replication of *P. falciparum* pIDNA initiates within the inverted repeat (IR) region that covers a ∼10 kb segment and carries genes for large and small subunit rRNAs and several tRNAs [10]. Using complementary approaches of electron microscopy and two-dimensional gel analysis, Williamson et al. [1] proposed that *P. falciparum* pIDNA replication proceeds via two mechanisms. The first and predominant mechanism, also inferred by us [2], follows the theta mode with replication initiating within the IR region while a minor population of pIDNA molecules may follow the rolling circle mode possibly utilising as yet unidentified initiation sites outside the IR. Using 5′ end-labelled nascent DNA to probe different segments within the IR, we now demonstrate the presence of multiple ori within each IR sector. Measurement of potential ori activity by competitive PCR additionally suggests differential utilization of initiation sites during *P. falciparum* pIDNA replication.

2. Materials and methods

2.1. Parasite culture

*P. falciparum* (strain NF-54) was cultured in human RBCs maintained in RPMI-1640 supplemented with 0.5% AlbumaxII (Invitrogen). The parasites were synchronised with sorbitol [23] and harvested at the late trophozoite-early schizont stages.

2.2. Preparation of pIDNA and enrichment of 5′ end-labelled replication intermediates

pIDNA-enriched DNA was purified using the QIAGEN plasmid mini preparation kit as described earlier [2]. Briefly, parasites released from infected RBCs by saponin lysis were washed with PBS, suspended in buffer P1, lysed with buffer P2, and neutralised with buffer P3. After centrifugation, the sample was treated with Proteinase K and passed through an equilibrated Qiagen tip-20 column. The column was washed with PBS, suspended in buffer P1, lysed with buffer

2.3. Southern hybridization

PlDNA sequences cloned in plasmid constructs [2] were digested with restriction enzymes, electrophoresed on 0.8 or 1.4% agarose gels and blotted onto nylon membrane using standard procedures [25]. 5′ end-labelled nascent DNA (‘caffeine wash’) was used as probe for hybridization. The membranes were washed and exposed to X-ray film for autoradiography. Densitometric analysis was carried out using ImageMaster 1D Elite software (Amersham Biosciences).

2.4. Purification of nascent DNA for competitive PCR

Total *P. falciparum* DNA was isolated from cultures using 10 T75 flasks at ∼10% parasitemia containing parasites at the late trophozoite-early schizont stages. Total parasite DNA was isolated by phenol/chloroform extraction [26]. Isolation of nascent DNA was carried out by sucrose gradient fractionation followed by further size selection of the fractionated DNA by agarose gel electrophoresis [27–29]. Total parasite DNA was denatured by a 10 min incubation in boiling water and size separated on 16 ml of 5–30% continuous neutral sucrose gradient (∼150 µg of DNA per gradient) for 18–20 h at 26,000 rpm in a Beckman SW28 rotor at 15 °C. Sucrose gradients were prepared in 10 mM Tris–HCl (pH 8), 1 mM EDTA, and 0.3 M NaCl. The bottom of the tube was punctured and 500 µl fractions were collected from each tube. Fractions containing 0.3–1.5 kb segments of nascent DNA were pooled and dialysed against Tris-EDTA [0.5 M Tris (pH 8), 0.01 M EDTA] for at least 8 h. DNA was precipitated with sodium acetate and ethanol, rinsed with 70% ethanol, dried, and suspended in TE. Further size selection was performed by fractionating the nascent DNA on a 1% preparative agarose gel and eluting 0.3–1.5 kb segments of DNA from the gel. After purification, the concentration of this DNA was determined and the preparation was used as template in competitive PCRs.
2.5. PCR amplification and competitor construction

Primers used for competitor construction and competitive PCR analysis of the 325 and 251 bp ori regions as well as primers used to amplify the 332 and 317 bp control non ori regions are shown in Table 1. Competitor construction for each of these regions was carried out as described by Diviacco et al. [30], and Habib and Hasnain [29]. Four specific oligonucleotides (two external primers, P1 and P2, and two internal primers, P3 and P4) were synthesized for each of these regions within which the point of equivalence between competitor and control regions is to be amplified. The upper (P4) and lower (P3) internal primers have 5' ends identical to contiguous sequences on the upper and lower genomic strands, respectively, and their 5' ends carry a 20 nt tag. The 20 nt tags of the P4 and P3 primers are complementary to each other and unrelated to the target sequence to be amplified. Competitor DNA segments carrying the corresponding genomic sequence with the addition of 20 extra bp in the middle were constructed for each primer set in a three-step process [29]. One or more subsequent reamplification steps of the full-length competitor were needed to enrich for the competitor product and allow its quantification by radiolabelling. All amplification reactions were carried out in an advanced version of thermostarII thermal cycler [31].

Competitor template for each DNA region was quantified by measuring the amount of incorporated [α-32P]dATP in a competitor reamplification PCR cycle. The competitor PCR amplification mixture (50 μl) contained the standard amount of cold dATP (10 mM) and 0.2 μl (0.57 pmol) of [α-32P]dATP (Jonaki, India) (3500 Ci mmol−1 and 10 μCi ml−1), corresponding to 1.34 × 10^7 cpm as measured by Cerenkov counting in a β-counter. The amplification products were resolved on an 8% polyacrylamide gel, and the labeled competitor band was eluted in 100 μl of water. Five microlitres of the eluted DNA was counted and the concentration of the competitor (number of molecules per microlitre) was determined from the final specific activity of [α-32P]dATP and the number of nucleotides incorporated. Dilutions of this quantified competitor preparation were used as template in competitive PCRs.

2.6. Competitive PCR experiments

Competitive PCR experiments were carried out for each region by first using a fixed amount of nascent DNA template with 10-fold serial dilutions of the corresponding competitor in the presence of primers P1 and P2. The range within which the point of equivalence between competitor and template lay was thus determined. Further dilutions of competitor within this range were then used in similar PCR reactions. Competitive PCR for the 325 bp (region I) and 251 bp (region II) segments was carried out in 30 cycles with the following conditions: denaturation, 94 °C, 1 min; annealing, 47 °C, 1 min; extension, 72 °C, 1 min. PCR conditions for amplification of the control non ori regions III and IV (332 and 317 bp, respectively) were as above except that the annealing temperature for the 332 bp region was 41 °C.

3. Results and discussion

3.1. Each segment of the IR contains at least three replication origins

The use of BND-cellulose, that binds single-stranded nucleic acids more strongly than double-stranded DNA, to enrich for DNA molecules containing replication intermediates...
Fig. 1. Schematic representation of the location of A650, A1820, and A1988 in a sector of the \textit{P. falciparum} plDNA inverted repeat. Restriction sites of relevant enzymes on plDNA as well as fragments generated by digestion of cloned plDNA IR sequences are indicated. Level of signal obtained in 5' end-labelling experiments is indicated by '+' or '-' signs. Speckled region in the plDNA marks the zone of replication initiation. The location of large subunit rRNA (LSU), small subunit rRNA (SSU) and tRNAs is shown.

Fig. 2. Hybridisation with \textit{NspI}-digested, end-labelled nascent DNA indicates the presence of at least three ori within each sector of the IR. (i) Agarose gel showing plDNA clones digested to release fragments A650, A1820 and A1919 as well as A650 digested with \textit{NspI}. A1919 is a control non-ori plDNA region. (ii) Southern hybridization of the same gel with \textit{NspI}-digested 5' end-labelled DNA gave a signal in the 417 bp fragment of A650 (indicated by arrow). The signal observed above this band is from undigested A650 that can be seen in the ethidium bromide-stained gel as a faint band. Markers (M) are shown in base pairs.

In order to determine whether the signal obtained for A650 was due to 5' end-labelled nascent DNA molecules emanating from the overlapping 1820 region, we digested labelled nascent strands of plDNA with \textit{NspI} and used these to probe A650 (previously cloned by us \cite{2}) that had also been digested with the same enzyme (Fig. 2). \textit{NspI} cuts at the edge of the overlapping region of A650 and A1820 (Fig. 1) so that molecules emanating from A1820 and entering the rest of A650 would be cleaved by the enzyme. Southern hybridization gave a signal for the 417 bp fragment of A650 while no signal was detected for the 232 bp fragment (Fig. 2) indicating that the 417 bp segment of A650 contains an independent ori. The presence of at least three ori within each sector of the IR can thus be inferred.
3.2. The IR serves as a replication initiation zone with multiple origins exhibiting differential activity

To determine the location of ori sequences within the A1820 and A1988 segments we digested cloned 1988 and 1820 segments as well as the 5′ end-labelled nascent DNA probe with combinations of different restriction enzymes (Fig. 1). The signals obtained in Southern blots were analyzed to determine approximate ori locations as well as relative ori efficiencies.

When labelled nascent DNA digested with ScaI and ClaI (cleavage with Scal would prevent entry of molecules from the A1820 region while ClaI would cut within A1988) was used to probe A1988 digested with ClaI, signals were obtained for both the 1257 and 730 bp fragments (Fig. 3 A). The relative intensity ratio of the signals was 1:1.16 (A1257:A730) indicating the presence of initiation sites within both fragments of A1988. Control plDNA fragments A1919 and A1288 that have previously been shown to lack ori [2] did not give a signal. A1988 was also digested with HindIII and probed with 5′ end-labelled DNA digested with HindIII and HincII (Fig. 3 B). A strong signal was observed only for the 1277 bp fragment while no signal was obtained for the 542 bp fragment of A1820 (Fig. 3 B, panel ii). The 1277 bp fragment digested with HincII gave signals in both the 740 and 537 bp fragments while the 542 bp fragment of A1820 lacks an ori. A portion of this 542 bp fragment overlaps with the 232 bp fragment generated by the digestion of A650 with NspI that also did not give a signal (Fig. 2). Additionally, no signal was obtained in the 542 bp fragment generated by digestion of A1820 with HindIII and probed with end-labelled plDNA digested with HindIII and Hhal (data not shown). Unlike the rest of the IR, this region is deficient in sequences capable of forming strong hairpin loops, a feature of most replication origins. The presence of a strong signal in the 1277 bp fragment of A1820 is in agreement with results of Williamson et al. [1] who detected a bubble pattern in this region in two-dimensional gels. They suggested the presence of an ori in the 1026 bp Hhal–Scal region of each sector of the IR which is cov-
3.3. Competitive PCR analysis confirms differential ori activity within two IR regions

To confirm whether ori sequences throughout the IR were differentially utilized during initiation of plDNA replication we adopted the method of competitive PCR that has been used for mapping ori in mammalian cells [27,32,33] as well as the determination of abundance of sequences within ori regions in nascent DNA preparations [28,29]. A fixed amount of DNA sample enriched in nascent DNA (i.e., low molecular weight DNA emanating from ori) is coamplified with increasing amounts of a quantified reference template (competitor) so that the two templates compete for the same primer set and amplify at the same rate. The ratio between the final amplification products of the two species is a precise reflection of the ratio between the initial amounts of the two templates and is used to evaluate the amount of the unknown nascent DNA template. This method of quantification of nascent DNA has shown a high level of sensitivity and fidelity for sequences that are believed to be at or near ori [28,34]. The isolation of nascent DNA in the size range of 0.3–1.5kb ensures maximal elimination of broken genomic parental DNA and large nascent DNA fragments including sheared DNA (typically ranging from 25 to 50kb) as well as Okazaki fragments (25–300nt) from replication forks.

Competitor DNA fragments were constructed for two IR ori regions (I and II) as well as two non-ori control regions (III and IV) of P. falciparum plDNA (Table 1 and Fig. 4). The two ori regions selected for the experiment lie within the 1277 bp and 973 bp fragments of the A1820 and A1988 segments, respectively. These regions had given high signals in 5’ end-labelling experiments described above. The non-ori control regions III and IV lie within plDNA segments A1919 and A1880, both of which have been previously reported to lack ori activity [2] (Table 1 and Fig. 4). A fixed amount of nascent DNA isolated from parasites at the late trophozoite-early schizont stage was added to the PCR mix with increasing amounts of the corresponding competitor DNA. The ratio of the competitor and template reaction products (C/T) was calculated after densitometric analysis of the ethidium bromide-stained gels and was plotted against the number of competitor molecules added to each reaction (Fig. 5). The number of competitor molecules when C/T = 1 was calculated from the plot equation. This value corresponds to the precise number of molecules of the target template (nascent DNA) added to the PCR. Competitive PCR analysis carried out for regions I and II revealed that region II was represented by ~3 times higher number of molecules than region I (6 × 10^4 molecules representing region II and 1.9 × 10^4 molecules representing region I) (Fig. 5A and B). No nascent DNA template amplification product was obtained for control non-ori regions (III and IV) although the primers amplified corresponding fragments from total parasite DNA preparations (Fig. 5C) indicating that DNA sequences from these regions were not represented in the nascent DNA sample prepared by us. The difference in number of nascent DNA molecules representing regions I and II suggests that although replication initiation sites around both sequences are utilized during plDNA replication those near region II are more active.

Although the mechanism by which unit-length, circular genomes of P. falciparum plDNA are generated is not fully understood there is substantial evidence to indicate that a bidirectional ori/D-loop mechanism is the primary mode of plDNA replication with a minor population of plDNA molecules following a rolling circle mode [1,2,18]. Previous identification of two replication initiation sites within the

![Fig. 4. Location of ori regions I and II and non-ori control regions III and IV in P. falciparum plDNA (diagram not to scale). The IRa sector of plDNA is shown with relevant restriction enzyme sites as well as positions of A1920 and A1988.](image)
Fig. 5. Competitive PCR reveals greater abundance of sequences derived from region II in plDNA nascent DNA preparations. Determination of the number of molecules representing regions I and II are shown in panels A and B, respectively. A fixed amount (1 µl) of nascent DNA template was added to competitive PCRs for the two regions. PCR products were resolved on an 8% polyacrylamide gel and stained with ethidium bromide. The intensity of the bands corresponding to the template target (T) and competitor (C) was determined by densitometric analysis. The ratio between the two PCR products for each reaction (C/T) was plotted against the number of competitor molecules added to the reaction. A linear correlation between the C/T ratio and the quantity of competitor added to the reaction was observed. Correlation coefficients (R²) are reported for each plot. The number of target template molecules, that equal the number of competitor molecules when C/T = 1, was calculated from the equation of the line fitting the experimental points. Panel C shows results of PCR amplification of control non-ori regions III and IV and ori region I from a nascent DNA preparation (lanes 3, 5, and 7) and total genomic DNA (lanes 2, 4, and 6).

each IR sector of plDNA had led us to suggest parallels with Oenothera chloroplast replication where two D-loops in each IR have been identified [2]. However, evidence for the presence of multiple initiation sites within each IR sector now implies that the IR serves as a replication initiation zone where more than two ori in each sector may be utilized at the same time. Moreover, differential levels of activation of replication ori observed by us indicates that all ori may not be utilized for replication of a plDNA molecule and that there may be a certain degree of redundancy in ori requirement. The identification and characterization of origin-binding proteins in the apicoplast would be the next step towards understanding the mechanism of ori activation and initiation of replication of P. falciparum plDNA.

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