Differential association of Orc1 and Sir2 proteins to telomeric domains in *Plasmodium falciparum*

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Summary

Telomeres have the capacity to recruit proteins that facilitate the spreading of heterochromatin into subtelomeric DNA regions. In the human protozoan pathogen *Plasmodium falciparum*, the telomere-associated protein Sir2 has been shown to control the silencing of members of virulence genes at some, but not all, chromosome-end loci, indicating that additional proteins are involved in telomere position effect. Here, we identified, in *P. falciparum*, a novel telomere-associated protein that displays homology with the origin-of-recognition-complex 1 protein Orc1. Antibodies raised against this *P. falciparum* protein localized to telomeric clusters in the nuclear periphery and the nucleolus. It was found that, prior to DNA replication, *P. falciparum* Orc1 and Sir2 undergo drastic subcellular reorganization, such as dissociation from the telomere cluster and spreading into the nucleus and parasite cytoplasm. Relocation of Orc1 and Sir2 was also linked to the partial dissociation of telomere clusters. Super gel-shift and chromatin-immunoprecipitation experiments showed the physical association of Orc1 with telomere repeats but revealed a differential association with adjacent non-coding repeat DNA elements. Our data suggest that *Plasmodium* telomeres might fold back and that Orc1 cooperates with Sir2 in telomeric silencing.

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Introduction

Telomeres are essential protein-DNA structures that protect all chromosome ends from degradation and fusion, and are vital for complete replication of chromosomes (Blackburn, 2006). In several organisms, telomeres are anchored to the nuclear periphery and recruit a number of proteins that initiate and/or facilitate heterochromatin formation into the adjacent subtelomeric regions (Hediger and Gasser, 2002). In yeast, as in higher eukaryotes such as humans, genes positioned near to telomeric repeats are silenced by a phenomenon called telomere position effect (TPE) (Baur et al., 2001; Gottschling et al., 1990). This form of epigenetic silencing is best characterized in budding yeast, and involves mainly Rap1 and Sir (silent information regulator) proteins, which assemble in a step-wise manner. Rap1 binds to telomeric repeats and recruits Sir2 via interaction with Sir4. Sir2 deacetylates nucleosomes that favour the binding of Sir3 and Sir4. Multiple rounds of deacetylation and binding of Sir complex along the nucleosomes lead to heterochromatin propagation and silencing at the proximity of telomeres. This spreading extends for ~3 kb from the telomere (Mondoux and Zakian, 2006). A rather unusual TPE was observed recently in the human malaria parasite *Plasmodium falciparum*. In this unicellular protozoan pathogen, heterochromatin spreads from telomeres and adjacent non-coding subtelomeric repeats over 20-30 kb into coding regions, thereby establishing gene repression on different chromosome ends (Duraisingh et al., 2005; Freitas-Junior et al., 2005).

P. falciparum telomeres, which are composed of degenerate G-rich heptamer repeats (5′-GGGTT[T/C]A-3′), form clusters of four to seven heterologous chromosome ends at the nuclear periphery (Freitas-Junior et al., 2000). Telomere repeats are followed by a mosaic of six different telomere-associated repetitive elements (TAREs 1-6), which are always found in the same order but vary in size (Figueiredo et al., 2000; Gardner et al., 2002). This subtelomeric region is important to maintain chromosome ends clustered in the nuclear periphery and to regulate telomere length (Figueiredo et al., 2002). The proteins that crosslink telomeres, however, remain unknown (Marty et al., 2006). Members of the *var* multigene family are located adjacent to the non-coding TAREs. *var* genes encode a key virulence factor expressed at the surface of infected red blood cells, which is strongly linked to malaria pathogenesis in humans (Kyes et al., 2001). The sequential expression of different members of the *var* gene family in a mono-allelic fashion (antigenic variation) leads to immune escape and chronic infection of the parasite in its human host (Craig and Scherf, 2001).

We previously identified orthologues to several yeast telomere-associated proteins in the *P. falciparum* genome (Scherf et al., 2001) and characterized in detail a protein homologous to the yeast Sir2 (Duraisingh et al., 2005; Freitas-Junior et al., 2005). *P. falciparum* Sir2 is a telomeric protein that reversibly associates with the promoter regions of silent but not active subtelomeric *var* genes (Freitas-Junior et al., 2005). Mutant-Sir2 parasites derepress *var* genes; however, transcription of only a subgroup of this gene family is upregulated (Duraisingh et al., 2005), indicating that TPE is a rather complex process involving additional chromatin factors in malaria parasites.

In this work, we identified a protein associated with telomeric and subtelomeric heterochromatin in *P. falciparum*. This molecule has been suggested to be the origin-of-recognition-complex 1 molecule (Orc1) of *P. falciparum* parasites (Gupta et al., 2006; Li and Cox, 2003; Mehra et al., 2005). Here, we show that *P. falciparum* Orc1 localizes specifically with distinct perinuclear sub-compartments: the telomeres and the nucleolus. In addition, we show that telomeric clusters disassemble prior to DNA replication, which is linked to *P. falciparum* Sir2 and Orc1 relocation within the nucleus and to the cytoplasm. Our findings indicate that nucleo-cytoplasmic shuttling of telomere-associated proteins might reveal novel functions of these molecules.

Results

Sir2 and Orc1 colocalize to telomeric and nucleolar regions

In order to search for putative factors involved in TPE in *P. falciparum*, we previously identified a sequence homologous to the yeast telomere-associated silencing factor Sir3 (Scherf et al., 2001). We generated antibodies against the putative *P. falciparum* Sir3 recombinant GST fusion protein. These antibodies recognized a band of 130 kDa in *P. falciparum* nuclear blood-stage extracts (supplementary material Fig. S1). Subsequent analysis revealed that the open reading frame identified is now annotated in the *P. falciparum* database (www.plasmodb.org) as being homologous to the origin recognition complex 1 (Orc1) protein (PFL01050w). In fact, Sir3 and Orc1 amino acid sequences are very similar in budding yeast because *SIR3* resulted from duplication of the *ORC1* gene (Kellis et al., 2004). Orc1 has been found in yeast subtelomeric regions (Pryde and Louis, 1999; Wyrick et al., 2001) and in other transcriptionally repressed domains, such as the mating-type loci in yeast (Bell et al., 1993) and the pericentric heterochromatin in *Drosophila* (Pak et al., 1997), where it participates in heterochromatin assembly. This led us to hypothesize that Orc1 is involved in telomeric silencing in *P. falciparum*. To test whether *P. falciparum* Orc1 localizes to telomeric heterochromatic regions, we performed immunofluorescence assays in which parasites were double-labelled using anti-Orc1 and -Sir2 antibodies. We observed that more than 80% (n=104) of the Orc1 foci colocalized with the Sir2 signals (Fig. 1A), indicating that Orc1 localizes to telomeres. However, Sir2 is not an exclusive marker for telomeric clusters but also stains the nucleolus (Freitas-Junior et al., 2005). We used anti-Nop1 antibodies as a nucleolar marker to test whether Orc1 also localizes in the nucleolus. A fraction of Orc1 signals indeed colocalized with the region defined by Nop1 (Fig. 1B). We confirmed the association of Orc1 to the telomeres and the nucleoli by immunofluorescence/fluorescence in situ hybridization (IF-FISH) using telomeric (TARE6) (supplementary material Fig. S2) and nucleolar (rRNA) probes (data not shown), respectively.

Our results show that, at ring stage, Orc1 localizes mainly at the nuclear periphery rather than within the entire nucleoplasm. We also show that it localizes to similar compartments as Sir2, supporting the idea that Orc1 might participate in heterochromatin formation in *P. falciparum*.

Sir2 and Orc1 proteins spread differentially into subtelomeric repeats

To study the binding of Sir2 and Orc1 to telomeric and subtelomeric domains in *P. falciparum*, we performed mobility-shift and supershift assays (EMSA). These assays were carried out using nuclear extracts and specific radioactively labelled probes directed against telomere, TARE3 and TARE6 (Fig. 2A). In the mobility shift assay, the probes for telomere, TARE3 and TARE6 formed retarded complexes, indicating that telomeres and subtelomeric repeats contain elements specifically recognized by nuclear proteins. Telomere showed a more complex pattern, suggesting that distinct protein complexes can bind to these sequences. The specificity of these complexes was confirmed using 50-fold molar excess of either homologous or heterologous competitors (Fig. 2B).

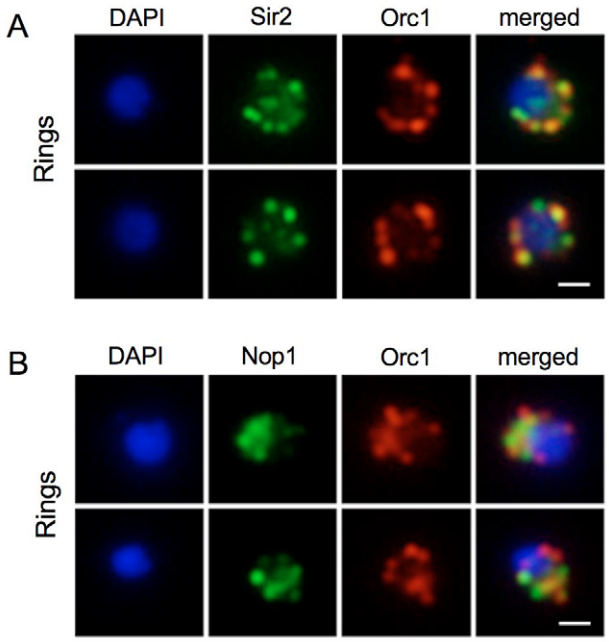

Fig. 1. Orc1 localizes to telomeric foci and the nucleolus. (A) Double IF using anti-rabbit Orc1 (red) and anti-rat Sir2 (green). Orc1 is preferentially localized at the periphery of the parasite nucleus and colocalizes with Sir2 signals. (B) Dual-colour IF by using anti-rabbit Orc1 (red) and anti-rat Nop1 (green). A fraction of Orc1 signals colocalize with Nop1, suggesting that Orc1 also localizes in the nucleolus of the parasite. (A,B) Parasites are in ring stage and nuclear DNA was stained with DAPI (blue). Scale bars: 1 μm.

Anti-Sir2 antibodies produced supershifted complexes with telomere, TARE3 and TARE6 probes. Anti-Orc1 antibodies formed a supershifted complex only with telomere and TARE3 (Fig. 2C). These results demonstrate that Sir2 and Orc1 bind specifically to telomeric and subtelomeric repeats. Moreover, the data suggest that Orc1 spreads from the telomere to TARE3, whereas Sir2 expands from telomeres to TARE6.

We used chromatin immunoprecipitation assays (ChIP) to further investigate the in vivo association of Sir2 and Orc1 with telomeric and subtelomeric chromatin. For this mapping study, we used DNA probes for telomere repeats, TARE1, TARE2, a sequence between TARE2 and TARE3, TARE 3, and TARE6 (Fig. 2A). We found that Orc1 and Sir2 proteins are present at the telomere, TARE1-3 and TARE6 (Fig. 3A,B). This confirmed most of the EMSA data, with the difference that the ChIP experiments show that Orc1 was also enriched in TARE6. The observed difference in Orc1 location might be explained by the fact that EMSA does not take into consideration possible particular telomere architecture that could bring Orc1 from the telomere into the TARE6 region by a fold-back structure. Telomeres are known to form fold-back structures in yeast (de Bruin et al., 2000; de Bruin et al., 2001; Strahl-Bolsinger et al., 1997; Zaman et al., 2002) and the same was proposed recently for *P. falciparum* (Figueiredo and Scherf, 2005).

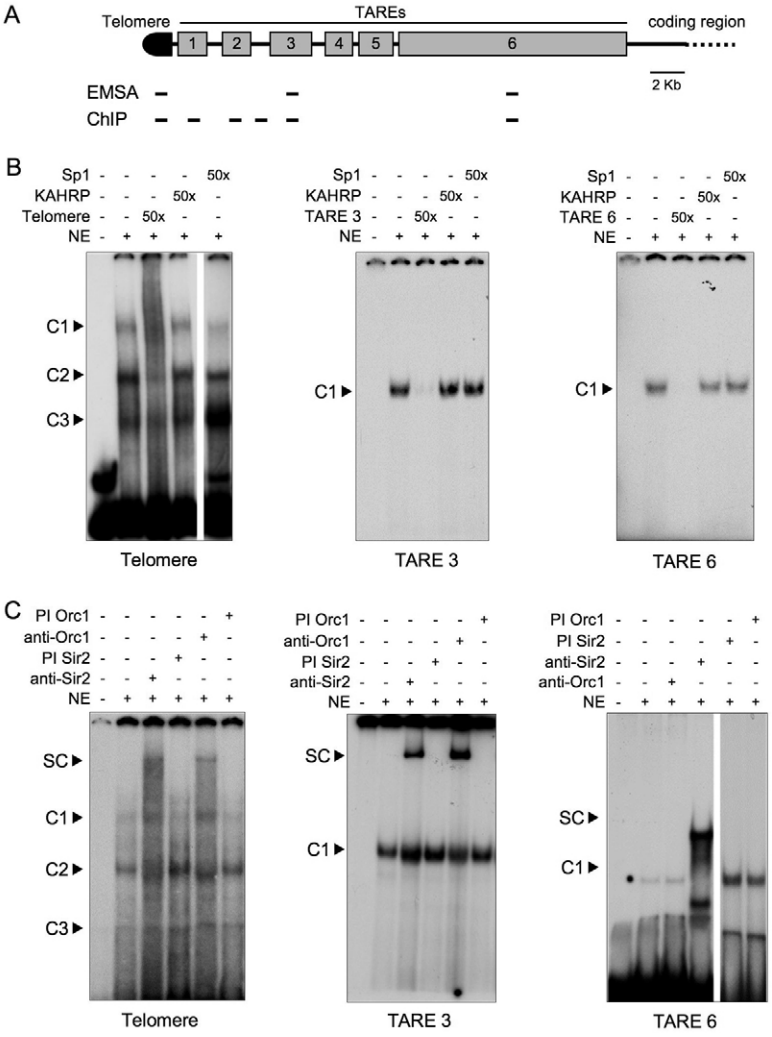

Fig. 2. Orc1 and Sir2 specifically recognize elements on the telomeric and subtelomeric repeats. (A) Schematic representation of telomere and subtelomeric regions of *P. falciparum* chromosome ends. The probes used in the EMSA and ChIP assays are indicated. (B) Binding of nuclear proteins to telomeric and subtelomeric regions. ^{32}P-labelled telomere (left panel), TARE3 (middle panel) and TARE6 (right panel) probes were incubated with nuclear extracts. Competition shift assays were done using either 50-fold molar excess of unlabelled homologous (third lane) or heterologous (KAHRP and Sp1, fourth and fifth lanes) competitor. A free probe was run in the first lane in all cases. The telomere probe formed three protein-DNA complexes, whereas TARE3 and TARE6 formed only a single retarded complex. Arrowheads indicate DNA-protein complexes. (C) Binding of Orc1 and Sir2 to telomeric and subtelomeric repeats. A total of 30 μg of antiserum against Sir2 and Orc1, and respective non-immune sera, were pre-incubated for 15 minutes in the binding reaction, followed by the addition of the labelled telomere, TARE3 and TARE6 probes. Telomere and TARE3 probes produced supershifted complexes with both anti-Sir2 and -Orc1 antibodies (left and middle panels), whereas the TARE6 probe only formed a supershifted complex in the presence of Sir2 (right panel). NE, nuclear extracts; C, complex; SC, supershifted complex; KAHRP, upstream region of *kahrp* gene; Sp1, consensus Sp1-binding-site factor; PI, pre-immune serum.

Sir2 and Orc1 relocate during the blood-stage cycle

During the ~48-hour blood-stage cycle, the parasite matures through the ring, trophozoite and schizont stages, and undergoes multiple rounds of asynchronous nuclear division (Leete and Rubin, 1996). Because Orc1 was assumed to be involved in DNA replication (Li and Cox, 2003; Mehra et al., 2005), we sought to analyze whether Orc1 localizes through S-M phase (trophozoite and schizont stages). We collected parasites from a synchronized culture at three different time points: rings, trophozoites and schizonts (Fig. 4A). Double-labelling IF studies revealed that, as the parasite differentiates, there is an increase in Sir2 and Orc1 protein levels together with their relocalization to a non-nuclear region (Fig. 4B-D). Strikingly, the IF pattern changes from well-defined perinuclear spots in ring stages to rather diffuse small dots in trophozoite stages. Moreover the colocalization of Orc1 and Sir2 (>80% in ring stage) decreases as the parasite matures.

DNA synthesis in *P. falciparum* can be reversibly blocked by aphidicolin, an inhibitor of DNA-polymerase-α in eukaryotes (Inselburg and Banyal, 1984). To understand the redistribution of Sir2 and Orc1 throughout the cycle, we cultivated the parasites in medium containing aphidicolin for about 24 hours. This culture, composed of arrested late trophozoites (Fig. 4A), was collected and subjected to IF analysis. Orc1 localized inside the nucleus in non-replicative trophozoites, which is consistent with its

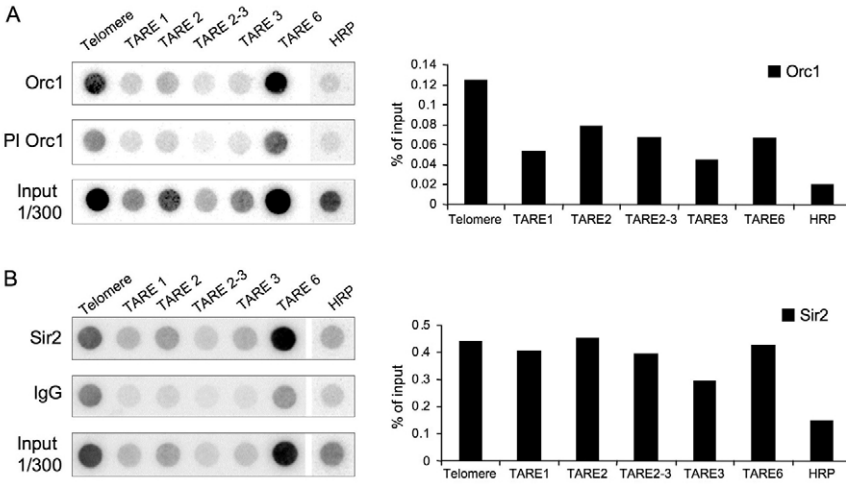

Fig. 3. Orc1 and Sir2 distribution at telomeric and subtelomeric chromatin. (A,B) ChIP analysis of ring-stage parasites using antibodies against Orc1 (A) and Sir2 (B). Immunoprecipitated DNA was analyzed by dot-blots hybridized with probes specific to telomeric sequences, subtelomeric sequences (TARE1, TARE2, TARE2-3, TARE3 and TARE6) and HRP. A representative dot-blot is shown for each antibody (left panels). The right panels show a quantitative presentation of the data shown in the left panels. In all cases, ChIP values represent a percentage of the total input DNA after subtraction of the background signal value (i.e. the material immunoprecipitated by the pre-immune sera or IgG). Association of Orc1 with telomeric and subtelomeric repeats is similar to that of Sir2.

putative replicative function. In addition, we observed a punctate and diffuse pattern outside of the nucleus. Surprisingly, Sir2 displayed a similar IF staining pattern (Fig. 4E). We concluded that reorganization of Orc1 and Sir2 occurred prior to DNA replication. To identify the subcellular localization of Sir2 and Orc1 in mature stages, an anti-Hsp70 antibody was used as a cytoplasmic marker (Mattei et al., 1989). Sir2 (Fig. 4F) and Orc1 (data not shown) colocalize with Hsp70 in aphidicolin-treated parasites. Sir2 and Orc1 are thus partially released to the cytoplasm in mature stages.

In order to exclude possible IF fixation artefacts, we performed double-IF assays in which we used anti-Sir2 and -histone-H3 antibodies. Clearly, histone H3 remained associated with nuclear DNA, whereas Sir2 relocated to the cytoplasm during parasite maturation (Fig. 4G). We also tested an alternative fixation protocol described previously (Tonkin et al., 2004), and the same

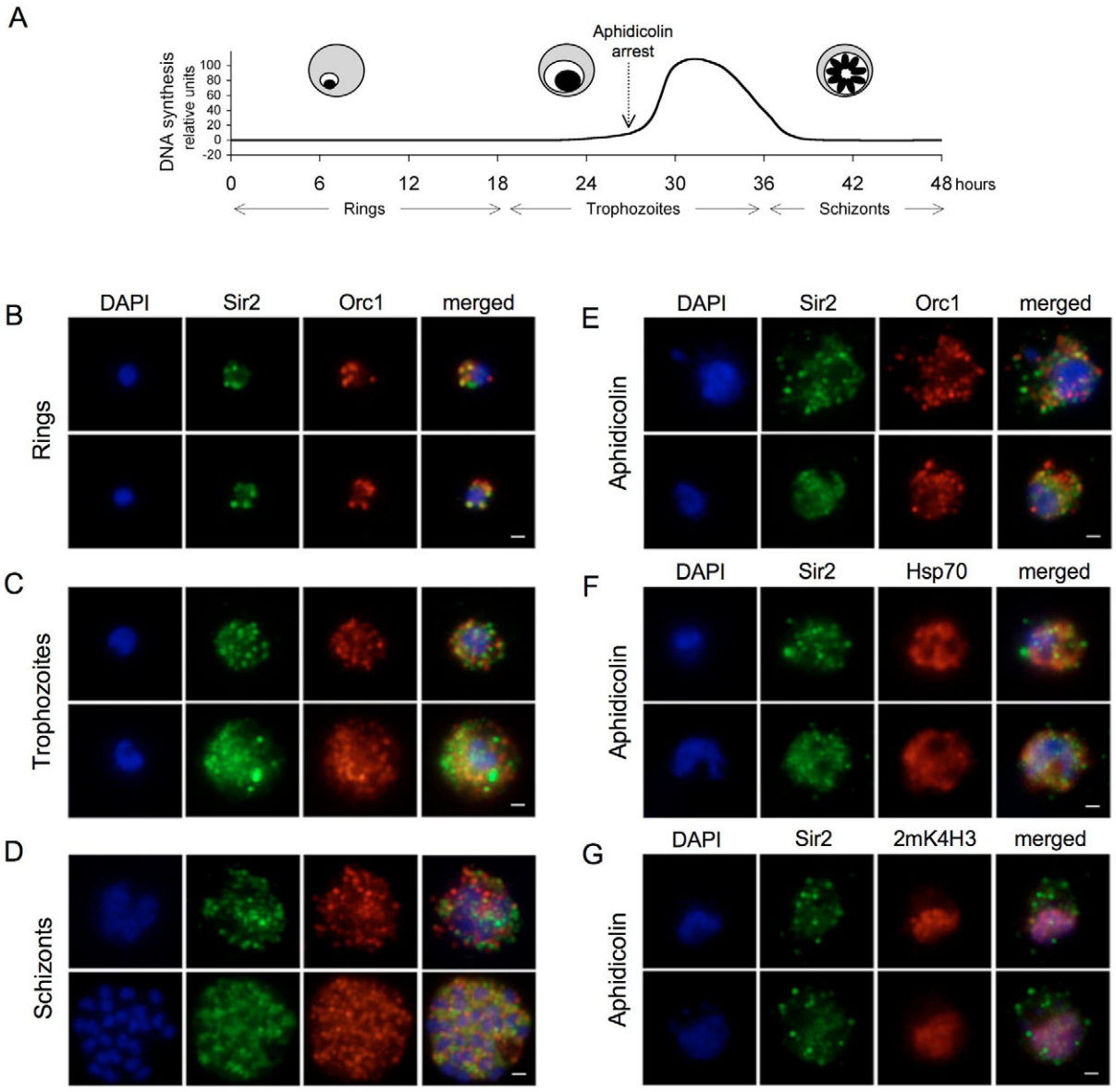

Fig. 4. Sir2 and Orc1 relocalize during the developmental cycle of *P. falciparum*. (A) DNA synthesis during the *P. falciparum* cell cycle. During the 48-hour blood-stage cycle, the parasite differentiates through ring [between 0- and 18-hours post-invasion (p.i)], trophozoite (18- to 36-hours p.i.) and schizont (36- to 48-hours p.i.) stages. DNA replication in *P. falciparum* takes place in the trophozoite stage; it peaks at 30 hours and can be blocked by adding aphidicolin to the culture medium. Nuclear division occurs by schizogony, leading to the production of 16-32 merozoites; these are released in the bloodstream and can initiate a new cycle by invasion of a new red blood cell. (B-D) IF analysis of Sir2 (green) and Orc1 (red) during the blood-stage cycle. (B) Ring stages display a punctate pattern at the nuclear periphery. (C) In the trophozoite stages, anti-Sir2 and -Orc1 antibodies reveal an apparent increase of both protein levels, and an additional punctate and diffuse pattern inside and outside of the nucleus. (D) The schizont stage, Sir2 and Orc1 seem to relocalize at the nuclear periphery. (E-G) Double-labelling IF using parasites cultivated with aphidicolin for ~24 hours. (E) Aphidicolin-treated parasites demonstrate that the redistribution of Sir2 (green) and Orc1 (red) occurs prior to initiation of DNA replication. (F) Labelling with an anti-mouse Hsp70 (red) revealed that Sir2 (green) is displaced into the cytoplasm of the parasite before S-phase. (G) Histone H3 (dimethyl K4, 2mK4H3; red) remained associated with the nucleus. Nuclei were detected by DAPI staining (blue) in all the figures. Scale bars: 1 μm.

Orc1 and Sir2 redistribution pattern was observed (data not shown).

Telomeric clusters break up before DNA replication

Chromosome ends of *P. falciparum* form four to seven telomeric clusters at the nuclear periphery (Freitas-Junior et al., 2000). In this previous work, only pre-replicative-stage parasites were analyzed. Considering the relocation of the telomere-binding proteins to the cytoplasm, we studied whether the telomeric clusters were rearranged in the nucleus of the parasite through its maturation. To address this issue, we analyzed rings, trophozoites and schizonts by FISH. Telomeres were visualized by using a fluorescein-labelled DNA probe corresponding to TARE6 (or rep20). We observed an increase from 4.3±0.3 signals per nucleus (n=64) in rings (Fig. 5A) to 12.5±1.2 (n=59) in trophozoite stages (Fig. 5B). Schizont stages displayed more than 30 FISH signals per parasite. It was difficult to determine the exact number of FISH dots because of overlapping signals of densely packed 16 to 32 nuclei (Fig. 5C). We also assessed the number of telomeric clusters in the non-replicative aphidicolin-arrested parasites. Unexpectedly, we scored 10.0±1.0 FISH signals (n=56), indicating that the telomeric clusters are partially disrupted before DNA replication (Fig. 5D). It is interesting to note that these nuclei show both weak and strong signals, and also that some of the telomeres seem to be dislocated from the nuclear periphery.

Discussion

P. falciparum telomeres and their adjacent subtelomeric regions have been described as being important elements in the nuclear positioning of chromosome ends and in the control of expression of virulence genes involved in antigenic variation and pathogenesis (Ralph and Scherf, 2005). Although the finding of Sir2 has shed some light on epigenetic control of antigenic variation in this pathogen (Duraisingh et al., 2005; Freitas-Junior et al., 2005), it also taught us that a single telomeric protein is not sufficient to control repression of all subtelomeric *var* genes. In this study, we report the Orc1 protein as another telomere-associated protein in *P. falciparum*. EMSA and ChIP studies clearly demonstrate that Orc1 can interact specifically with telomere and with various subtelomeric repeats in a similar way as Sir2 (Figs 2 and 3), pointing to Orc1 as a strong candidate molecule that contributes to telomeric silencing in *Plasmodium*. Remarkably, Orc1 appears to have retained the replication and silencing functions observed in other eukaryotes (Chesnokov, 2007).

In addition, our EMSA and ChIP results suggest that *Plasmodium* telomeres fold back, allowing the telomeric chromatin to interact with the subtelomeric domains, as shown schematically in Fig. 6A. This fold-back structure might account for the stabilization of telomeric and subtelomeric chromatin at the nuclear periphery in *Plasmodium*, as previously suggested in yeast (de Bruin et al., 2000; Strahl-Bolsinger et al., 1997).

Interestingly, the Orc1 N-terminal region has a leucine zipper motif and many charged amino acid residues potentially involved in DNA-protein and protein-protein interactions (Li and Cox, 2003; Mehra et al., 2005). In our model (Fig. 6A), we hypothesize that the putative N-terminal DNA-binding motif allows the direct binding of Orc1 to the telomeric DNA. By contrast, Sir2 association to telomeric and subtelomeric chromatin probably involves other molecules, because Sir2 has no evident DNA-binding motifs. Although no interaction between Orc1 and Sir2 was reported in the

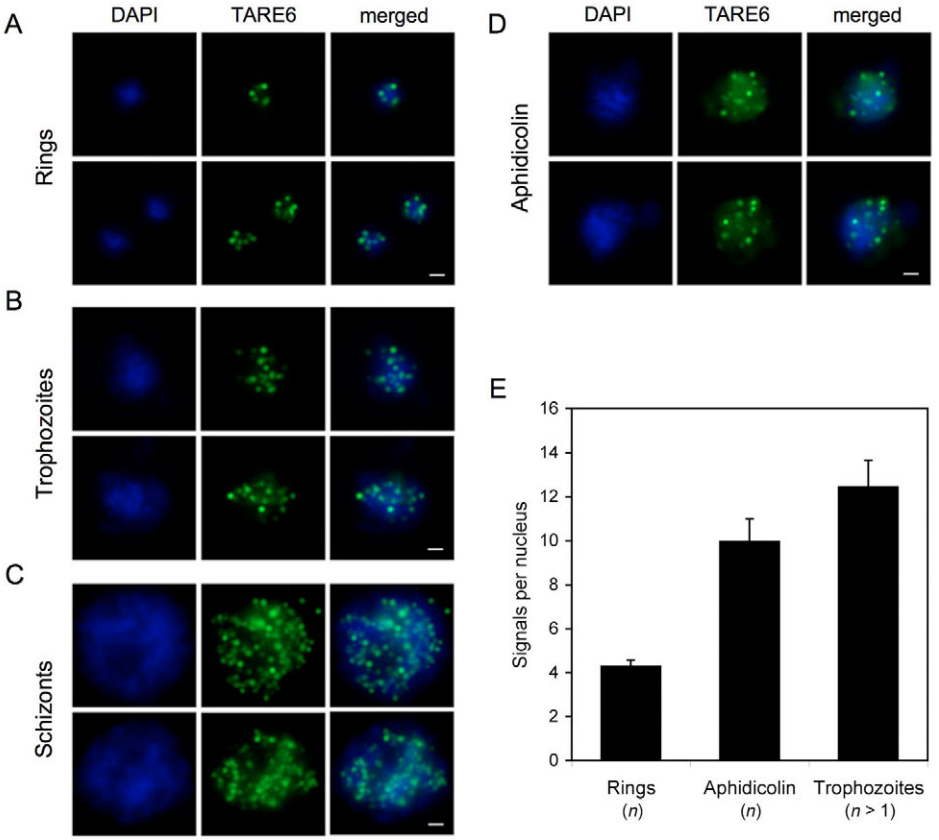

Fig. 5. Visualization of the telomeric clusters during the blood-stage cycle. (A-D) FISH analysis of nuclei stained with DAPI (blue) and hybridized with TARE6 (green) probe to visualize the chromosome ends on rings (A), trophozoites (B), schizonts (C) and aphidicolin-treated parasites (D). Scale bars: 1 μm. (E) Quantification of TARE6 FISH signals in rings, trophozoites and aphidicolin-treated parasites. *n* refers to the chromosome number per nucleus; in trophozoite stage, $n>1$, depending on the number of nuclear divisions that occurred on each individual parasite. In the case of aphidicolin-treated parasites, all signals (weak and strong) were counted. Error bars are 95% confidence intervals (±1.96 s.e.m.). The standard deviations for rings, aphidicolin-treated parasites and trophozoites are 1.20, 3.93 and 4.71, respectively.

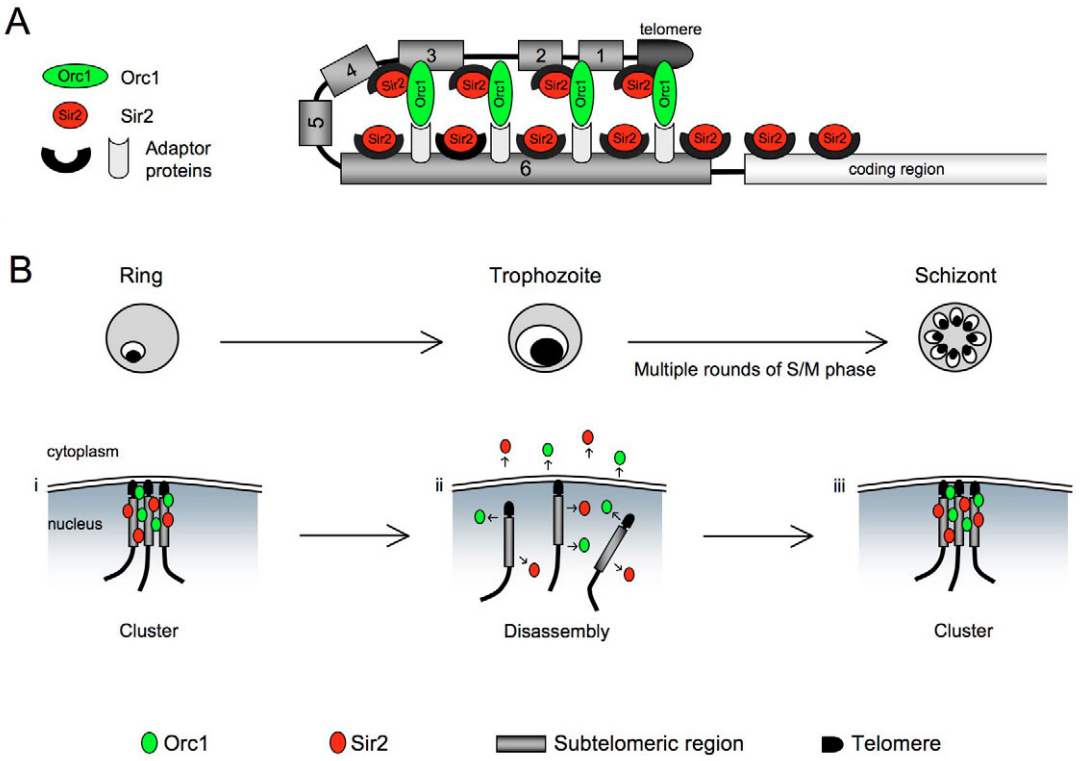

Fig. 6. Model for dynamic telomeric heterochromatin assembly and relocation during blood-stage development. (A) Hypothetical model of the differential spreading of *P. falciparum* telomeric proteins. Orc1 (green ovals) might bind directly to telomere and to TARE1-3 repeats via its N-terminal DNA-binding domain. The interaction of Orc1 with other unknown TARE6-binding molecules (adaptor proteins) might lead to telomere bending. Sir2 (red circles) might be recruited via interaction with an unknown telomere-associated protein (black curve) or alternatively with Orc1. Sir2 might then deacetylate telomeric histone tails (Merrick and Duraisingh, 2007), promoting heterochromatin formation and spreading towards the coding region. (B) Model for the dynamics of telomere chromatin factors during the *P. falciparum* blood-stage cycle. During ring stage (left), parasite telomeres form clusters at the nuclear periphery and associate with Sir2 (red circles) and Orc1 (green circles) (i). This period (G phase) is followed by multiple rounds of DNA synthesis and nuclear mitosis (trophozoite stage, middle), which produce a multinucleate schizont (right). Our data show that Orc1, Sir2 and telomeric clusters disassemble prior to DNA replication (ii). Telomeric components assemble in the newly formed nuclei (iii) and will be maintained for the next cycle. We speculate that the relocation events are driven by specific post-translational modifications. Alternatively, cytoplasmic Orc1 and Sir2 might correspond to newly synthesized proteins necessary to accommodate the demands of the rapid nuclear divisions occurring during S-M phase.

yeast two-hybrid screening assay (LaCount et al., 2005), Orc1 might work as the initiator of heterochromatin assembly and be involved in Sir2 recruitment into the telomere tract. Apart from Sir2, the other proteins of the Sir complex (Sir1, Sir3 and Sir4) are not present in this parasite. It has been suggested that the lack of the Sir complex might be compensated for by the presence of the heterochromatin protein 1 (HP1) (Moazed, 2001). Strikingly, the orthologue to HP1 has been recently identified in *P. falciparum* (K. Toledo and R.H.-R., unpublished data). ORC-HP1 interactions have been described in *Drosophila* (Pak et al., 1997), *Xenopus* (Pak et al., 1997) and mammals (Auth et al., 2006; Prasanth et al., 2004) in centromeric heterochromatin, implicating HP1 as another candidate protein that interacts with Orc1 in *Plasmodium* telomeres. Taking into account all this data, it is becoming evident that the molecular components contributing to TPE in *P. falciparum* resemble more the silencing complexes in metazoans and fission yeast, rather the budding-yeast complexes.

Unexpectedly, Orc1 colocalized with Sir2 not only in the telomeric clusters but also in the nucleolus (Fig. 1). In yeast, Sir2 also localizes in the nucleolus (Gotta et al., 1997) and represses both recombination and polymerase-II-dependent transcription within the ribosomal (r)DNA repeats (Gottlieb and Esposito, 1989; Smith and Boeke, 1997). The function of Sir2 in the nucleolus has not been determined so far. Sir2-mutant parasites apparently do not change the transcription pattern of plasmodial rRNA genes (L.M.-S. and A.S., unpublished). Regarding Orc1, to our knowledge, no localization studies in other organisms have shown this protein to be present in the nucleolus. It is striking, though, that three proteins localizing in the nucleolus and having functions elsewhere in the nucleus have already been observed: TERT (Figueiredo et al., 2005), Sir2 (Freitas-Junior et al., 2005) and Orc1 (this work). An attractive hypothesis is that the nucleolus might serve as a reservoir for telomere-associated proteins, as has been proposed for yeast Sir proteins (Gotta et al., 1997).

One of the most remarkable features of Orc1 and Sir2 is that they undergo dynamic relocations throughout the 48-hour blood-stage cycle in *P. falciparum*. They localize to distinct perinuclear compartments after merozoite invasion (ring stage) all the way through early trophozoite stage (20-hours post-invasion), and reposition to the entire nucleus and parasite cytoplasm during the late trophozoite stage, the part of the cycle in which schizogony takes place to generate between 16 and 32 nuclei (Fig. 4 and schematically shown in Fig. 6B). Nuclear mitosis in *Plasmodium* is asynchronous and occurs without cytoplasmic division (Leete and Rubin, 1996). DNA-replication machinery might cause the disruption of telomeric clusters, unfolding of telomeres, and

consequent dispersion of Orc1 and Sir2. Arresting the parasites by aphidicolin allowed us to take a picture of the nuclear organization of the parasite prior to S-M phase. This demonstrated, however, that Orc1 and Sir2 relocations occur before DNA replication and might be linked with the break-up of telomeric clusters (Fig. 5 and schematically shown in Fig. 6B). Cell-cycle-dependent relocalization and remodelling of silencing factors have also been reported for yeast (Laroche et al., 2000; Smith et al., 2003). However, Rap1 and Sir proteins partially disperse from the telomeric clusters only in G2 phase and mitosis (Laroche et al., 2000). This discrepancy between *Plasmodium* and yeast might be due to the unusual nuclear mitosis that occurs in this parasite.

Differential post-translation modifications might explain differential localizations, and might account for other functions of Orc1 and Sir2 at different time points of the *Plasmodium* blood-stage cycle. In fact, a recent study in our laboratory indicates that the nuclear form of Sir2 is modified by SUMO (N. Issar, E. Roux, D. Mattei and A.S., unpublished). The amino acid sequence of Orc1 has several SUMO consensus motifs, implying that Orc1 is also sumoylated. It is tempting to speculate that reversible sumoylation might cause the relocation of Sir2 and Orc1. Sumoylation has been described as regulating subcellular localization (Heun, 2007); it has also been shown that telomere-associated proteins are often sumoylated (Potts and Yu, 2007; Xhemalce et al., 2007), pointing to this as a feature conserved in evolution. Orc1 also contains phosphorylation sites in the N-terminus (Mehra et al., 2005), indicating that phosphorylation state might also dictate localization and activity of this protein.

In summary, this work reveals dynamic changes that occur in the perinuclear chromatin organization and telomere architecture during *P. falciparum* blood-stage differentiation and proliferation. Our study of Orc1 reveals another dimension of plasmodial chromosome ends, namely that, during blood-stage development, telomeres share the same molecule with other DNA elements, such as the origin-of-replication sequences and possibly rDNA, within the nucleolus. Future work should address directly the role of Orc1 in the nucleolus, cytoplasm and TPE in *P. falciparum*. TPE is of particular interest owing to its central role in the regulation of virulence-factor genes and antigenic variation in this parasite. Because ORC genes are essential for cell survival (Chesnokov, 2007) and because of the absence of an applicable inducible gene-knockdown system in *P. falciparum*, only mutations touching dispensable functions such as telomere silencing can be studied in this parasite; namely, mutations in the N-terminal region, because the function in DNA replication seems to reside exclusively in the C-terminus (Mehra et al., 2005). Further studies should clarify whether nuclear and cytoplasmic forms show different post-translational modifications and whether or not they are linked with the potential multiple functions of Orc1.

Materials and Methods

Parasites

P. falciparum parasites were cultivated according to standard conditions (Trager and Jensen, 1976). Aphidicolin (Sigma) was added to a synchronized ring-stage culture as described previously (Inselburg and Banyal, 1984).

Antibodies

A rabbit anti-Orc1 antibody was prepared by immunizing rabbits with a GST fusion protein corresponding to 150 amino acids on the C-terminus of the Orc1 protein (aa794-944). Rat sera against Sir2 and against Nop1 were obtained by immunizing rats with two synthetic peptides coupled to KLH for each protein. The sequence of the peptides is described elsewhere (Figueiredo et al., 2005; Freitas-Junior et al., 2005). Rabbit polyclonal antibody to histone H3 (dimethyl K4) was purchased from Abcam.

Immunofluorescence microscopy

Synchronized cultures of *P. falciparum* were washed in phosphate-buffered saline (PBS), lysed in saponine (0.015%) and fixed in suspension with 4% paraformaldehyde solution for 15 minutes. Parasites were then incubated with the primary antibodies diluted in 1% bovine serum albumin (BSA) at 37°C for 30 minutes. After washing, parasites were incubated at 37°C for 30 minutes with the secondary antibodies conjugated with fluorochromes. After final washes, parasites were deposited on microscope slides and mounted in Vectashield anti-fading with 4-6-diamidino-2-phenylindole (DAPI). Images were captured using a Nikon Eclipse 80i optical microscope.

Anti-Orc1 and anti-Sir2 antibodies were pre-absorbed with lysed non-infected red blood cells before incubation with the fixed parasites. The final antibody dilutions were: rabbit anti-Orc1 1:50-100, rabbit anti-Sir2 1:100, rat anti-Sir2 1:50, rat anti-Nop1 1:50, monoclonal mouse anti-Hsp70 1:1200, rabbit anti-H3 2mK4 1:200, Alexa-Fluor-488-conjugated goat anti-rabbit highly cross-absorbed 1:500, Alexa-Fluor-568-conjugated goat anti-rabbit highly cross-absorbed 1:500, Alexa-Fluor-568-conjugated goat anti-mouse highly cross-absorbed 1:500 and Fluorescein-conjugated goat anti-rat 1:500.

Fluorescence in situ hybridization

FISH was performed with the same lysed and fixed parasites used for the IF to allow a close correlation between IF and FISH imaging. Briefly, the fixed parasites were deposited on microscope slides, permeabilized in 0.1% Triton X-100 and hybridized with heat-denaturized TARE6 probe (see ChIP probes) at 80°C for 30 minutes and at 37°C overnight. After hybridization, slides were washed as described previously (Freitas-Junior et al., 2000).

For immunofluorescence combined with in situ hybridization (IF-FISH) parasites were prepared as described above for IF; incubation with secondary antibody, parasites were post-fixed in suspension with 4% paraformaldehyde solution for 15 minutes, deposited on microscope slides and followed for FISH, in the same conditions described above.

Nuclear extract preparation, gel-shift and super-shift assays

Preparation of nuclear extracts, gel shift and super-gel shift were performed as described previously (Freitas-Junior et al., 2005; Ruvalcaba-Salazar et al., 2005). Telomere probe of 175 bp was amplified from a plasmid obtained in a previous work (Figueiredo et al., 2000) using the primers listed in supplementary material Table S1. A 550-bp DNA fragment of TARE3 was amplified from pCR2.1TOPO-TARE3 (see ChIP probes); it was then digested with *Eco*RI and *Ava*II enzymes to get a TARE3 probe of 180 bp. TARE6 probe contains three Rep20 units and was obtained by hybridization of a 79-bp oligonucleotide with the respective anti-sense oligonucleotides (supplementary material Table S1). Kharp and Sp1 probes were obtained as described previously (Ruvalcaba-Salazar et al., 2005).

ChIP assay and dot-blots

The ChIP assay was performed as described previously (Freitas-Junior et al., 2005; Lopez-Rubio et al., 2007). Chromatin fragments were incubated overnight at 4°C with the following antibodies: 15 µl of rabbit anti-Sir2, 4 µl of rabbit anti-IgG, 10 µl of rabbit anti-Orc1 and the respective preimmune sera. The immunoprecipitated DNA was radioactively labelled and hybridized with a Hybond N+ membrane dot-blotted with 35 ng of telomeric, TARE1, TARE2, TARE2-3, TARE3 or TARE6 probes. TARE1, TARE 2-3, TARE3 and HRP were PCR amplified from genomic DNA using the primers listed in supplementary material Table S1 and cloned in pCR2.1-TOPO (Invitrogen). The telomeric probe was PCR amplified using the telomere primers (supplementary material Table S1) from the plasmid previously obtained (Figueiredo et al., 2000). TARE2 (2 kb) and TARE6 (1.5 kb) probes were amplified using the primers M13 from plasmids obtained in a previous work (Figueiredo et al., 2000).

For total DNA samples (input), an aliquot of lysate used in the immunoprecipitation was processed along with the rest of the samples. Signals were quantified using the ImageQuant software. Calculation of the amount of immunoprecipitated DNA in each ChIP was based on the relative signal to the corresponding total DNA signal.

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