International Journal for Parasitology 40 (2010) 35–43

Contents lists available at ScienceDirect

International Journal for Parasitology

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

The effect of *Plasmodium falciparum* Sir2a histone deacetylase on clonal and longitudinal variation in expression of the *var* family of virulence genes

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ARTICLE INFO

Article history:
Received 17 April 2009
Received in revised form 28 June 2009
Accepted 30 June 2009

Keywords:
Plasmodium falciparum
var
Sirtuin
Epigenetic
Silencing
Antigenic variation

ABSTRACT

Plasmodium falciparum, the most important causative agent of human malaria, undergoes antigenic variation as a means of prolonging infection and ensuring transmission between hosts. Clonal variation is observed in the surface adhesins expressed on infected erythrocytes: primarily in the *Pf*EMP1 adhesin encoded by the large *var* gene family. The sirtuin *Pf*SIR2A was the first protein discovered to have a major influence on antigenic variation in *P. falciparum*. In the absence of *Pf*SIR2A, normal silencing of the variantly-expressed *var* gene family is partially deregulated. To thoroughly investigate the role of *Pf*SIR2A in controlling antigenic variation, multiple independent clones of wildtype and *Pf*SIR2A-knockout (Δ*Sir2a*) parasites were generated. *var* gene expression was then measured qualitatively, quantitatively and longitudinally over extended periods in culture. Δ*Sir2a* parasites were found to activate about 10 specific *var* genes in every independent clone analyzed. The activated genes were biased towards the *upsA*, *upsBA* and *upsE* *var* gene subclasses. The total *var* transcript level was two to three-fold higher in Δ*Sir2a* parasites than in wildtype parasites and at least one transcript – encoding the pregnancy malaria adhesin VAR2CSA – was successfully translated and expressed on the infected cell surface. In the absence of *Pf*SIR2A, antigenic switching over time was also diminished, although not abolished. This work expands our understanding of clonal antigenic variation in this important human pathogen and demonstrates a central role for *Pf*SIR2A in regulating both the variant expression of specific *var* gene subsets and the overall quantity of *var* gene expression.

1. Introduction

Plasmodium falciparum is the most important cause of human malaria, giving rise to widespread morbidity and approximately 1 million deaths each year. This unicellular eukaryotic parasite lives and replicates inside erythrocytes and is spread between human hosts by a mosquito vector. To avoid the human immune system and sustain chronic infections, *P. falciparum* has evolved a complex system of antigenic variation, allowing it to persist in the bloodstream for months or years, thus facilitating insect transmission.

Antigenic variation in *P. falciparum* is best characterized in the major surface antigen expressed on infected erythrocytes, *Pf*EMP1 (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). This large transmembrane protein mediates adhesion to a variety of host endothelial molecules, sequestering infected cells out of the circulation to avoid splenic clearance. The exposed *Pf*EMP1 protein is, however, a target for host immunity (Bull et al., 1998) and a large family of variant '*var*' genes encoding antigenically distinct forms of *Pf*EMP1 has therefore evolved. There are 63 genes in the sequenced strain 3D7, with the majority located sub-telomerically and a subset in tandem arrays at chromosome-internal locations (Gardner et al., 2002). *var* genes have been divided into sub-families according to the similarity of their '*ups*' upstream promoter regions. *UpsB* genes are the most telomere-proximal and are transcribed towards the centromere; *upsA* genes are located sub-telomerically, inside the *upsB* genes and are transcribed towards the telomere, while *upsC* genes are chromosome-internal. Intermediate promoter types define *upsBA* and *upsBC* *var* genes. There is also a unique *upsE* gene encoding the unusual *Pf*EMP1 protein VAR2CSA, which adheres to the placental marker chondroitin sul-

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doi:10.1016/j.ijpara.2009.06.012

phate A (CSA) and is likely to play a major role in pregnancy malaria (Salanti et al., 2003; Viebig et al., 2005).

Most evidence suggests that only a single *var* gene (Scherf et al., 1998; Dzikowski et al., 2006; Voss et al., 2006) or a small subset of them (Duffy et al., 2002; Mok et al., 2007, 2008) is expressed in a single parasite at one time. The expressed gene(s) switches over time, which can correlate with a change in the adhesive phenotype of infected cells (Roberts et al., 1992) and with waves of fever in infected patients, as new parasite populations emerge which are not well controlled by the immune system (Miller et al., 1994). No defined orders for switching between *var* genes have been discovered in in vitro cultures, suggesting that the process is either highly complex or entirely random. It is also unclear how fast antigenic switching occurs and how widely the switch rate might vary, with published estimates ranging from 2% to 18% per generation (Roberts et al., 1992; Gatton et al., 2003).

The biological mechanism that controls transcriptional switching amongst *var* genes in *P. falciparum* has recently been the subject of intensive study. Switches occur without accompanying DNA rearrangements and an expressed gene is maintained semi-stably for multiple generations, implicating an epigenetic mechanism (Scherf et al., 1998). This idea was confirmed when a gene encoding a sirtuin in *P. falciparum* was genetically disrupted (Duraisingh et al., 2005). Sirtuins are NAD^+-dependent (class 3) deacetylase enzymes, named after the canonical *Saccharomyces cerevisiae* enzyme silencing information regulator 2 (*Sc*SIR2). *Sc*SIR2 is a crucial player in heterochromatin formation and thus in the general silencing of sub-telomeric genes, a phenomenon termed 'telomere position effect' in yeast. *Sc*SIR2 deacetylates histone tails, facilitating chromatin condensation and thus gene silencing. In accordance with the *S. cerevisiae* model, loss of the putative sirtuin *Pf*SIR2A in *P. falciparum* led to the activation of multiple *var* genes, particularly in sub-telomeric loci. In a complementary study, *P. falciparum* chromatin was found to be most condensed in sub-telomeric regions and semi-quantitative chromatin immunoprecipitation showed that histone H4 was less acetylated within a silent sub-telomeric *var* gene than within an active one. This hypoacetylation correlated with the presence of *Pf*SIR2A on chromatin (Freitas-Junior et al., 2005). The *Pf*SIR2A protein was subsequently shown to be a genuine sirtuin enzyme with deacetylase (and ADP-ribosylase) activity on *P. falciparum* histones in vitro (Merrick and Duraisingh, 2007). More recently, it has been shown that other histone modifications, notably methylations of histone H3, correlate with *var* gene expression or silencing (Lopez-Rubio et al., 2007) and a role has been demonstrated for the second sirtuin in *P. falciparum*, *Pf*SIR2B, in silencing a different subset of *var* genes (Tonkin et al., 2009).

Thus, *var* gene regulation appears to center on an epigenetic process which semi-stably silences the majority of the *var* gene family. *Pf*SIR2A plays an important role in controlling heterochromatinization, at least in sub-telomeric regions. Questions remain, however, as to the precise mechanism by which this enzyme influences antigenic variation, warranting a closer examination of the phenotype of *Pf*SIR2A-knockout (Δ*Sir2a*) parasites. For example, does *Pf*SIR2A normally silence a very specific subset of *var* genes – in which case each Δ*Sir2a* parasite should up-regulate the same large subset of genes simultaneously – or does it primarily influence switching, in which case there might be rapid switching amongst a preferred gene subset? Alternatively, might *Pf*SIR2A affect both the dynamics of switching and the nature of *var* expression at any one time? Are there common sequence features in the promoters of the activated *var* genes, pointing to a specific in-cis mechanism of transcriptional control? Finally, are the *var* RNAs that are over-expressed in Δ*Sir2a* parasites actually functional, being translated and expressed as a greater quantity and/or variety of *Pf*EMP1 on the infected cell surface?

To answer these questions, the phenotype of Δ*Sir2a* parasites was assessed both qualitatively and quantitatively in terms of *var* gene expression. Multiple independent clones of wildtype (WT) 3D7 and 3D7Δ*Sir2a* parasites were generated and their *var* expression profiles were measured and compared. Clones were then followed for a long period of time to assess switching. These experiments yielded, to our knowledge, the first detailed picture of global *var* expression dynamics, both in 3D7 WT parasites and as affected by the important silencing enzyme *Pf*SIR2A.

2. Materials and methods

2.1. Parasite culture

Plasmodium falciparum lines 3D7 (strain obtained from the Walter and Eliza Hall Institute of Medical Research, Australia), 3D7Δ*Sir2a* (Duraisingh et al., 2005) and CS2 (Elliott et al., 2005) were cultured in human O^+ erythrocytes at 4% haematocrit in RPMI 1640 supplemented with 0.5% albumax (Invitrogen) and 0.25% sodium bicarbonate, using standard procedures (Trager and Jenson, 1978). For flow cytometry experiments, cultures were switched from albumax to 10% human serum 48 h before collection. Cloning was achieved by limiting dilution (Kirkman et al., 1996). *var* expression profiles of clonal populations were assessed as soon as possible after cloning (15–24 generations), then at 45, 95 and 145 generations post-cloning. Approximately 2×10^8 ring-stage parasites were collected for all RNA preparations: synchronized rings were produced by treatment with 5% sorbitol.

2.2. Quantitative reverse transcriptase PCR (qRT-PCR) for var gene expression

Complementary DNAs (cDNAs) were prepared and assessed essentially as described in Dzikowski et al. (2006). Briefly, RNA was extracted from parasites using Trizol Reagent (Invitrogen), purified on PureLink columns (Invitrogen), treated with DNaseI (Invitrogen) and then reverse-transcribed using the Superscript II reverse transcriptase kit (Invitrogen). Each cDNA was checked for genomic DNA (gDNA) contamination using PCR across the intron of the gene PFD1155w, as described by Frank et al. (2007). qRT-PCR for *var* gene expression was carried out using the primer set designed by Salanti et al. (2003), with modifications as in Dzikowski et al. (2006) and Frank et al. (2007). The primer sets for the *var* genes PFB0010w and PF08_0107 were omitted, due to poor efficiency in our hands on 3D7 gDNA.qRT-PCR was carried out using ITaq SYBR Supermix (Bio-Rad) in an ABI Prism machine, as described in Dzikowski et al. (2006).

ΔΔCt analysis (Ct, threshold cycle) was used to calculate the relative copy number of each *var* gene, relative to the average Ct of the five control genes described in Dzikowski et al. (2006): PF07_0073 (seryl-tRNA synthetase), PFL0900c (arginyl-tRNA synthetase), PF13_0170 (glutaminyl-tRNA synthetase), PF14_0425 (fructose bisphosphate aldolase) and PFL2215w (actin). We also made the following correction, as described in Dzikowski et al. (2006), to adjust for slight differences in the Ct values obtained from gDNA using the *var* gene primer sets. qRT-PCR was carried out in duplicate on 3D7 gDNA and a ΔCt for each primer set was calculated relative to a single-copy housekeeping gene, PF07_0073. The correction factor for each individual *var* gene was then incorporated into the ΔΔCt analysis as follows:

ΔΔCt = {(Ct of target gene on cDNA – Ct of reference gene on cDNA)} – {(Ct of target gene on gDNA – Ct of reference gene on gDNA)}.

On and off rates of individual *var* genes were calculated as in Frank et al. (2007): $r_{on/off} = \Delta T/n$ where n is the number of genera-