Plasmodium falciparum Sir2: an Unusual Sirtuin with Dual Histone Deacetylase and ADP-Ribosyltransferase Activity

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In the human malaria parasite Plasmodium falciparum, a member of the sirtuin family has been implicated in the epigenetic regulation of virulence genes that are vital to malaria pathogenesis and persistence. This eukaryotic sirtuin, PfSir2, is divergent in sequence from those characterized thus far and belongs to the phylogenetic class that contains primarily eubacterial and archaeal sirtuins. PfSir2 cofractionates with histones in blood-stage parasites, and the recombinant enzyme efficiently deacetylates the N-terminal tails of histones H3 and H4. In addition, PfSir2 can ADP-ribosylate both histones and itself, an activity that is minimal or absent in most sirtuins with significant deacetylase activity. Strikingly, the deacetylase activity of PfSir2 is dependent on its ADP-ribosylation. Finally, although PfSir2 is not affected by established sirtuin inhibitors, it can be completely inhibited by nicotinamide, a natural product of the sirtuin reaction. This study shows that PfSir2 has the appropriate characteristics to be a direct regulator of chromatin structure in Plasmodium. It also raises the significant possibility that both ADP-ribosylation and deacetylation of histones could be sirtuin-regulated modulators of chromatin structure in this species.

Class III deacetylases are a family of NAD+-dependent deacetylase enzymes that are widely conserved from archaea to humans (21). They are also termed “sirtuins” after the founding member of the family, Saccharomyces cerevisiae ‘silencing information regulator 2’ (Sir2). ScSir2 plays a central role in epigenetic transcriptional control, acting as a histone deacetylase (HDAC) to establish regions of silent heterochromatin at subtelomeres, mating-type loci, and ribosomal DNA within the budding yeast genome (23). At least one of the seven sirtuins in humans, HsSirT1, appears to have a similar role in establishing gene silencing in human cells (61), suggesting that this function has been conserved through evolution. Many other sirtuins do not, however, act efficiently on histone substrates, including two additional nuclear sirtuins in humans (45), while others act on substrates in the cytoplasm or mitochondria. The roles of these sirtuins are generally not completely understood, but various members of the family have been assigned to processes such as longevity control (reviewed reference 26), DNA repair (22, 48), and the control of metabolic enzymes (27, 28).

All the sirtuin proteins crystallized so far have the same basic structure: a large NAD+–binding “Rossmann fold” domain and a smaller domain consisting of a zinc-binding module plus a variable, primarily helical, module (17, 46, 66). Sirtuins have a complex reaction mechanism compared to NAD+–independent (“class I and II”) deacetylases, involving the breakdown of an NAD+ cofactor to produce nicotinamide and O-acetyl-ADP-ribose as well as the deacetylated lysine product. This mechanism allows sirtuins to act as both deacetylases and ADP-ribosyltransferases (51). In fact, both ScSir2 and a human enzyme, HsSirT2, were initially shown to have ADP-ribosyltransferase activity rather than deacetylase activity: ScSir2 ADP-ribosylates both itself and its histone substrates, albeit at a very low level (20, 56). It was subsequently demonstrated, however, that a second, more significant activity, the deacetylation of histones H3 and H4, correlated with the effects of ScSir2 on transcriptional silencing and life span extension (34). It now appears that both deacetylation and ADP-ribosylation by different sirtuins are of biological importance, with certain sirtuins, such as ScSir2, being biased towards deacetylation and others, such as HsSirT6 and HsSirT4, towards ADP-ribosylation (27, 40).

In several eukaryotic microbial pathogens, including Plasmodium spp., variably expressed gene families encoding virulence determinants are located in potentially heterochromatic, subtelomeric regions of the genome. This observation suggests that there may be a common sirtuin-mediated mechanism for controlling antigenic variation in these pathogens (43). In the human malarial parasite Plasmodium falciparum, a sirtuin homolog, PfSir2, was recently shown to be involved in the epigenetic transcriptional control of a large number of subtelomeric genes that are vital for virulence (15). These included the multigene families var and rifin, both of which encode variably expressed antigens exposed on the surface of infected erythrocytes during a blood-stage malarial infection (11, 38, 54). When the PfSir2 gene was experimentally disrupted, mutually exclusive expression of var genes was abolished, and the expression of many var genes (and also some rifin genes) was simultaneously upregulated (15). In a complementary study, semiquantitative chromatin immunoprecipitation showed that H4 was more highly acetylated within an active subtelomeric var gene than within a silent one and that this hyperacetylation was mutually exclusive with the presence of PfSir2 (19). All of this evidence points to the idea that heterochromatic silencing,
mediated by PiSir2, could directly control the expression of var
virulence genes in *P. falciparum*.

In this study we have characterized the PiSir2 protein, es-
stablishing it as a genuine HDAC with the ability to act on
*Plasmodium* histones. In *P. falciparum*, the protein was found
in the parasite nucleus, where it cofractionated with histones.
Furthermore, PiSir2 could partially complement a sir2 mutant
in the heterologous species *Schizosaccharomyces pombe*. Strik-
ingly, in addition to deacetylation activity, PiSir2 had robust
ADP-ribosyltransferase activity. PiSir2 therefore possesses all
the necessary characteristics to contribute to the epigenetic
control of virulence genes in *P. falciparum*.

**MATERIALS AND METHODS**

**Recombinant protein preparation from *P. falciparum*.** *P. falciparum* was cul-
tured in human O+ erythrocytes using standard procedures (59). The PiSir2
gene was amplified by PCR from genomic DNA (*P. falciparum* 3D7 strain) and
cloned into an expression vector under control of the Pf
*hsa* promoter (65) with a C-terminal Hiss tag. Transformation into parasites was carried out as
previously described (64). *Saccharomyces cerevisiae* and mouse extracts of transformed par-
asites were made as described previously (63), using mixed-stage cultures at 3 to
5% parasitemia. For Ni-nitrilotriacetic acid (Ni-NTA) purification of PiSir2, parasite
extracts were made as described previously (63) but using a modified
buffer with sodium phosphate buffer replacing BSA, to act as a carrier. For high
resolution of histones, Tris-Tricine gels were used and stained with SYPRO
Ruby protein stain (Invitrogen) instead of Coomassie blue. For phosphodiester-
ase treatment, 5 µg phosphodiesterase (Sigma) was added to a completed reac-
tion in 100 nM Tris-HCl (pH 8.9), 100 mM NaCl, and 15 mM MgCl2; and then
incubated for 30 min at 37°C before TCA precipitation. For the repurification of
PiSir2 after such a treatment, 60 µg PiSir2 was incubated with 60 µg phospho-
diesterase for 2 h at 37°C in the above buffer, and the mixture was diluted five
times in 150 mM NaCl and then bound to Ni-NTA and eluted as before.

**RESULTS**

PiSir2 is a putative NAD^+^-dependent HDAC. PiSir2 is one
of the smallest eukaryotic sirtuins, having only 273 residues; it
is smaller than any of the *S. cerevisiae* or *Homo sapiens* sirtuins
(357 to 562 and 310 to 747 residues, respectively). In fact,
PiSir2 more closely resembles the *Archeaeoglobus fulgidus*
sirtuin AfI in size and overall sequence similarity. Moreover, a
classification of sirtuins from across the evolutionary spectrum
into four classes by molecular phylogeny places PiSir2 into
class III, the class containing AfI, many eubacterial sirtuins,
and only a single human homolog, the mitochondrial protein
SirT5 (21).

Figure 1 shows the predicted protein sequence of PiSir2
aligned with sirtuin sequences from archaea, yeast, and human
for which the encoded proteins have been crystallized and
structures solved. The alignment also includes a sirtuin from
*Plasmodium vivax* and the recently characterized sirtuin from
*Tryptosoma brucei*, TbSir2RP1. The core sirtuin domains of
PiSir2 are relatively poorly conserved with the human, yeast,
and *Archeoglobus* sirtuins (23 to 35%), but there is nevertheless
identity or similarity between PiSir2 and the extensively stud-
ed *S. cerevisiae* sirtuin Hst2 at all but one of the residues
known to be important for catalytic activity (36) (Fig. 1). For
example, the two pairs of cysteines required for zinc binding
(Cys140, Cys143, Cys168, and Cys170 in PiSir2) remain
conserved, and it has previously been noted that considerable
diversity is tolerated within the zinc-binding module outside of
these residues (17). PiSir2 is also broadly conserved at the
most invariant sirtuin motifs, the residues of which form an
NAD^+^-binding pocket in the AfI crystal structure (2). Here, it
has only three substitutions: GAGXS/GSGSX, GIPXFR/NIP
XFR, and TQNDXL/TQNYDXL.
PfSir2 has several changes at residues implicated in inhibition of the deacetylation reaction by nicotinamide. Of the residues that form a nicotinamide-binding pocket in Hst2 (50), Glu is changed to Arg62 and Phe to Gly65 in PfSir2. This phenylalanine is also altered in the Af1 and Af2 proteins, to alanine, possibly pointing to an altered potential for nicotinamide inhibition among all these class III enzymes. Similarly, most of the residues which contact an acetyl-lysine substrate in a ternary complex of Hst2, substrate, and product (67) remain conserved in PfSir2, although there are two conservative substitutions, Val to Ile179 and Leu to Val185, as well as one nonconservative change, Pro to Thr214. This could influence any binding of PfSir2 to acetyl-lysine substrates, altering the efficiency or specificity of this putative HDAC enzyme. Interestingly, most but not all of the alterations described here are also present in the P. vivax sirtuin homolog, which shares 80% similarity with PfSir2.

Outside of the core enzymatic domain, the N and C termini of the larger eukaryotic sirtuins are thought to mediate protein-protein interactions and/or regulate activity. For example, the N- and C-terminal domains in Hst2 have both been assigned functional roles: the C-terminal helices self-interact with the NAD⁺-binding site, causing autoinhibition, and this region also bears a nuclear export signal which keeps the enzyme primarily cytoplasmic and limits its nuclear deacetylase activity. The N-terminal seven residues of Hst2 mediate trimerization by interacting with, and apparently blocking, the substrate-binding cleft of an adjacent molecule (64, 66). Figure 1 shows that PfSir2 has a small N-terminal extension similar in length to that of Hst2, which may therefore be sufficient to play a functional role. The C terminus of PfSir2, however, is almost as short as that of the very minimal A. fulgidus sequence.

In order to support some of this in silico analysis with experimental evidence, we sought to express a recombinant PfSir2 protein.

**Production and characterization of recombinant PfSir2.** The PfSir2 gene was cloned from *P. falciparum* genomic DNA into a vector containing a C-terminal His₆ tag and a strong *P. falciparum* SP6 promoter. The resulting plasmid was transformed into *Escherichia coli* strain BL21 (DE3). The recombinant protein was purified by affinity chromatography on Ni⁺-NTA resin, followed by gel filtration. The purified protein was used for further characterization.

*Fig. 1.* PfSir2 aligned with a selection of crystallized sirtuins from other species. The predicted sequence of PfSir2 is aligned with the sequences of *A. fulgidus* Af1, *S. cerevisiae* Hst2, *S. pombe* Sir2, *H. sapiens* SirT2, *T. brucei* SIR2RP1, and the predicted homolog in *P. vivax*, PvSir2. Strictly conserved and conserved residues are shaded in black and gray, respectively. Starred residues are essential for catalytic activity in Hst2 (36), and where necessary, essential residues with slightly different spacings in different sirtuins are boxed to indicate their presence.
**P. falciparum** promoter, the Pfhs86 promoter (65) (Fig. 2A). This was transfected as an episome into the 3D7 strain of *P. falciparum*, and biochemical fractionation of transgenic parasites showed that tagged PfSir2 was in the insoluble chromatin-containing fraction together with the great majority of histones (Fig. 2B). This suggests that PfSir2 has the appropriate location for an HDAC enzyme and is bound to chromatin (or possibly another insoluble nuclear component), either directly or via protein-protein interactions. PfSir2 was resistant to extraction with 800 mM KCl, possibly pointing to a hydrophobic protein-protein interaction. Limited amounts of soluble PfSir2 could, however, be released using modified buffer conditions, and when this protein was purified from parasite extract via Ni-NTA, it eluted as a series of multimers that were at least partially resistant to SDS-PAGE (Fig. 2C).

In order to produce greater quantities of recombinant PfSir2, the gene was expressed in *E. coli* with a C-terminal His₆ tag and an N-terminal T7 tag. The recombinant protein was produced in a soluble form with the expected size of ~30 kDa, purified via Ni-NTA, and eluted at high purity (judged from SDS-PAGE and Coomassie blue staining) (Fig. 2D). Western analysis and mass spectrometry confirmed that the major, upper band of this doublet was indeed full-length, N- and C-terminally His₆-tagged PfSir2 (Fig. 2E).

The H₃ and H₄ tails are almost completely conserved between *P. falciparum* and *H. sapiens* (44), and PfSir2 effectively
deacetylated H3 and H4 from either source (Fig. 3C). PfSir2 generally appeared to prefer H3 as a substrate over H4, and *P. falciparum* histones were deacetylated more efficiently, particularly in the case of H3, than commercial preparations of HeLa histones. Previous studies of other sirtuins have reported significant preferences for particular acetylated residues, especially Lys16 of H4 and Lys9 of H3, which may be important components of the histone code relating to epigenetic silencing. It was not possible to establish conclusively whether PfSir2 preferred a single residue in H3 because the antibody recognized both AcLys9 and AcLys14, while an antibody specific for AcLys14 does not react well with *P. falciparum* histones (44), perhaps due to a single residue change at position 12, Gly to Ala. However, since the overall AcH3 tail signal was readily reduced to near-undetectable levels, both Lys9 and Lys14 are clearly deacetylated efficiently.

In regard to H4, there are four potentially modified lysines in the N-terminal tail, Lys5, Lys8, Lys12, and Lys16. Again, it was not possible to conclusively determine a preference for a particular residue because only antibodies to AcLys8 and AcLys12 gave detectable signals. PfSir2 had no activity on AcLys8 and only a very limited activity on AcLys12 (data not shown). It may, however, act more strongly on AcLys5 or AcLys16.

**PfSir2 is inhibited by nicotinamide but not by other established sirtuin inhibitors.** We next investigated whether the deacetylase activity of PfSir2 could be specifically inhibited with small molecules. Several inhibitors and activators of ScSir2 have been identified, including the inhibitors nicotinamide (5), sirtinol (25), and splitomicin (3) and the activators isonicotinamide (52) and resveratrol (33). All of these were tested on PfSir2 using the in vitro assay with substrate A.

Nicotinamide is a natural product of the NAD<sup>+</sup>-dependent deacetylase reaction (Fig. 3A), which can promote a back reaction (“nicotinamide exchange”) at the expense of the forward reaction (53). This compound only partially inhibited PfSir2 in the synthetic-substrate assay, reaching a plateau at ~65% of the uninhibited reaction rate (Fig. 4A). Strikingly, however, it inhibited the deacetylation of native histones much more effectively, with a high level of 10 mM being completely inhibitory (Fig. 4B) and even much lower levels (25 to 500 μM) showing substantial dose-dependent inhibition (Fig. 4C).

By contrast, splitomicin and sirtinol failed to inhibit PfSir2 in either assay (Fig. 4B and data not shown). Both drugs were tested at 50 μM, a concentration reported to reduce the reaction rate of a sensitive sirtuin, HsSirT1, by ~40% for splitomicin and ~70% for sirtinol (5). Indeed, both drugs at this concentration substantially inhibited ScSir2 in the same assay.
Even at 400 μM, splitomicin did not inhibit PfSir2 (data not shown); the same test cannot be carried out with sirtinol due to its precipitation in aqueous solutions. The failure of splitomicin to affect PfSir2 may be due to the lack of conservation between PfSir2 and ScSir2 (the sirtuin for which splitomicin was initially discovered) in the region that is thought to contact the drug (Fig. 4D). Hst2, which also lacks homology in this region, is similarly insensitive to splitomicin (3). No target region has been reported for sirtinol, but it may likewise be effective only against the sirtuins most closely related to its initial target, ScSir2.

Finally, PfSir2 was also unaffected by the activators isonicotinamide and resveratrol (data not shown). Isonicotinamide can apparently bind to the nicotinamide-binding site of ScSir2 and specifically inhibit nicotinamide exchange (53), but this compound had little effect on the reaction rate of PfSir2. Likewise, resveratrol failed to activate the enzyme, a finding which may not be surprising as it has been reported that the in vitro “activation” of sirtuins by this drug is related specifically to the use of the Fluor-de-Lys synthetic substrate (35).

**PfSir2 partially complements a fission yeast sir2 mutant.** To test whether PfSir2 could serve as an active HDAC in vivo as well as in vitro, it was used to complement a sir2 mutant strain of the fission yeast Schizosaccharomyces pombe. This strain carries the URA4 marker gene in a pericentromeric locus under Sir2-mediated silencing control. In the mutant, the URA4 gene cannot be silenced and a toxic prodrug, 5-FOA, can therefore be metabolized, killing the yeast. The addition of a functional sirtuin rescues this strain by silencing the URA4 gene; such successful cross-complementation was previously demonstrated when ScSir2 was expressed in S. pombe (18).

A PfSir2 construct was transformed into both wild-type and sir2 mutant S. pombe strains. When the yeast was grown on plates containing no thiamine, the overexpression of PfSir2 was toxic, an effect also observed with high levels of ScSir2 (18). Under conditions of thiamine repression and more moderate PfSir2 expression, however, the complemented strain grew 5- to 10-fold better on 5-FOA than an empty-vector control (Fig. 5).

**PfSir2 has ADP-ribosyltransferase in addition to HDAC activity.** Most of the sirtuins characterized thus far have either deacetylase or ADP-ribosyltransferase activity but are not significantly bifunctional. A sirtuin from Trypanosoma brucei, however, was recently reported to perform both functions ro-

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**FIG. 4.** PfSir2 is inhibited by nicotinamide but not by other established sirtuin inhibitors. (A) The deacetylase assay using substrate A as for Fig. 3B was carried out in the presence of 25 μM to 10 mM nicotinamide. Residual activity in nicotinamide-treated reactions is expressed as a percentage of the fluorescence released in the uninhibited reaction. The graph shows the averages from three assays, each carried out in triplicate. Error bars indicate standard deviations. (B) Western blots as in Fig. 3C, showing P. falciparum histones incubated with recombinant PfSir2 in the presence (+) or absence (−) of 500 μM NAD+, with 10 mM nicotinamide (NAM), 50 μM splitomicin (Split), or 50 μM sirtinol (Sirt). (C) Western blots showing P. falciparum histones incubated for 1 h with recombinant PfSir2 in the presence (+) or absence (−) of 500 μM NAD+ and then with NAD+ and also increasing levels of nicotinamide (25 to 10,000 μM). Densitometric quantification of the AcH3 signal in the absence of NAD was set at 1, and all subsequent signals were compared to this. Each value was corrected for the ratio of total H3 signal in each lane to total H3 signal in the NAD+ lane. Quantification of Western blot signals is linear only over a limited range, however, so values are at best semiquantitative over the whole range shown on this blot. (D) Alignment of the splitomicin target region of ScSir2 with the splitomicin-sensitive sirtuin Hst1 and the insensitive sirtuins Hst2 and PfSir2. Arrows mark residues which can be mutated to confer splitomicin resistance in ScSir2. None of these are conserved in PfSir2.
bustly, ADP-ribosylating both histones and BSA, as well as itself, and also deacetylating histones (22). This sirtuin, TbSir2RP1, falls into phylogenetic class I, and it is not very closely related to PfSir2 in sequence (Fig. 1); it is, however, another sirtuin from a protozoan parasite which, like P. falciparum, has relatively few sirtuin genes (4).

When PfSir2 was tested for the ability to ADP-ribosylate either BSA or Plasmodium histones using radioactive NAD\(^+\), significant activity was detected on both substrates (Fig. 6A, lanes 3 and 4). This was genuinely dependent on PfSir2, since a high level of nicotinamide inhibited the activity (Fig. 6A, lane 5), and substrate proteins were not significantly modified in the absence of the sirtuin (Fig. 6A, lanes 1 and 2). PfSir2 also ADP-ribosylated itself and was able to do this in the absence of other proteins (Fig. 6A, lane 6). Self-modification was, however, promoted by the presence of additional protein substrates, since the sirtuin became more strongly labeled when incubated in the presence of BSA and histones than when incubated alone. All four major histones became modified (see Fig. S3 in the supplemental material) but there was some preference for ADP-ribosylation of H2A, H2Bv, and/or H3 over H2B and H4. Figure 6B shows that the ADP-ribosyltransferase activity of PfSir2 was very robust compared to that of another sirtuin, Hst2 (which nevertheless has robust deacetylase activity). Finally, treatment with phosphodiesterase, which cleaves the pyrophosphate bond in ADP-ribose, was used to confirm that the labeling of PfSir2 was definitely due to the presence of \([^{32}\text{P}]\text{ADP-ribose}\) on the protein. This treatment did indeed reduce the intensity of the signal on radiolabeled PfSir2 (Fig. 6C).

Interestingly, prolonged treatment of recombinant PfSir2 with phosphodiesterase followed by repurification via Ni-NTA reduced the size of the major band of this protein. The size reduction was small but clearly visible on a highly resolved SDS-polyacrylamide gel (Fig. 6D), and concomitant with this, deacetylase activity was reduced by a factor of 10 (Fig. 6E). ADP-ribosylation of the major, upper band of recombinant PfSir2 therefore occurs when it is produced in *E. coli*, and the enzyme’s activity depends on this modification. It remains to be established whether this is because ADP-ribosylation is actually required to activate PfSir2—an unprecedented finding among sirtuins—or because artificial cleavage of the ADP-ribose moiety at the pyrophosphate bond is somehow inhibitory to deacetylase activity.

**DISCUSSION**

This report provides the first detailed characterization of an apicomplexan sirtuin, PfSir2. It is also one of the few reports published so far examining a sirtuin from phylogenetic class III, since most of the extensively studied human and yeast sirtuins fall into class I. Class III sirtuins appear to be a very ancient group, as they are the most frequently occurring sirtuins in both eubacteria and archaea (21). They have been maintained in some although not all eukaryotic species; humans, for example, possess a single class III sirtuin, SirT5. SirT5 is found in mitochondria, consistent with a possible endosymbiotic origin, and it is presently without an established function or substrates.

In silico analysis of the protein sequence of PfSir2 suggested that it was probably a functional sirtuin, possessing the great majority of residues known to be important for catalytic activity in the yeast sirtuin Hst2 and having broadly conserved NAD-binding motifs. *P. falciparum* also contains a second, larger sirtuin gene in phylogenetic class IV (21), but this gene remains unstudied and it is unclear whether the two sirtuins, both of which are predicted to be nuclear, could perform all of the functions shared between multiple sirtuins in several locations in other higher eukaryotes. Apicomplexans diverged very early in evolution, and other species in the phylum, including *Plasmodium* spp. and also *Toxoplasma gondii*, similarly have only two sirtuin genes, orthologous to those in *P. falciparum* (Fig. 1). Some sirtuin functions, such as tubulin deacetylation or the regulation of metabolic enzymes, may have been lost in the apicomplexan lineage, but specialized partner proteins may also have evolved to adapt the apicomplexan sirtuins to a number of different roles. ScSir2, for example, is able to act in at least two distinct genomic locations via its binding to different protein complexes (24, 57).

PfSir2 formed large multimers in solution, and a similar property has been observed in ScSir2 (57), so this may be a physiological feature of certain sirtuins. ScSir2 spreads along stretches of DNA when establishing silenced heterochromatin, and the ability to multimerize could perhaps facilitate such spreading. Alternatively, the multimeric form may simply be an artifact of overexpression or of preparation in the absence of any physiological protein partners and/or substrates. ScSir2, HsSirT1, and HsSirT2 can all be produced under certain conditions as stable homotrimers, and Hst2 exists in an equilibrium between trimeric and monomeric states, with substrate binding specifically disrupting the trimer (66). ScSir2 also preferentially forms heterodimers and heterotrtrimers with its heterologous binding partners Sir4 and Sir3 (12, 47, 61). No evidence was found in the present study for preferential trimerization of PfSir2, a property which could be unique to class I sirtuins.

When His-tagged PfSir2 was expressed in *P. falciparum*, the
protein was located in the insoluble chromatin-containing fraction of the parasite, together with the histones. PfSir2 may be bound here via hydrophobic interactions with a larger DNA-associated protein complex, perhaps containing many molecules of PfSir2 and being consistent with previous analyses of the large DNA-bound complexes containing ScSir2. ScSir2 does not itself have DNA-binding or nucleosome-binding activity (24), but it associates with chromatin via other proteins. The nuclear location of PfSir2 reported here is also consistent with a previous report that detected this protein in the parasite nucleus by immunocytochemistry (19). In the same report, regions of chromatin with highly acetylated histones were found to lack detectable PfSir2 and vice versa, pointing to a direct or indirect role for PfSir2 in histone deacetylation.

In this report, PfSir2 is directly shown to be an active HDAC. When its deacetylase activity was tested on two different synthetic substrates, PfSir2 clearly acted as an NAD-dependent deacetylase, although rather slowly, requiring overnight incubations to produce significant signals. PfSir2 also deacetylated Plasmodium histones, a probable physiological substrate, and this reaction could be completed to the limit of detection within 1 h under appropriate conditions. These assays are not directly comparable, but any disparity between them may occur because the synthetic substrates only poorly mimic optimal, physiological substrates for PfSir2 (the synthetic peptide, for example, is modeled on human p53, which has no clear homolog in P. falciparum). PfSir2 may also require an absent binding partner(s) for its optimal activity, as is the case for ScSir2 (12, 57).

When deacetylating histones, PfSir2 acted most strongly on the N-terminal tail of H3, which is acetylated in this species by the acetyltransferase PfGCN5 (16). By contrast, all of the class I sirtuins ScSir2, SpSir2, HsSirT1 and HsSirT2 act primarily on H4Lys16, although they also deacetylate H3Lys9 to various

FIG. 6. Recombinant PfSir2 has ADP-ribosyltransferase activity. (A) P. falciparum histones and/or BSA was treated with PfSir2 and [3H]NAD for 30 min at 37°C and then precipitated with TCA and subjected to SDSPAGE. The Coomassie blue-stained SDS-polyacrylamide gel shows the positions of PfSir2, histones, BSA, and a minor breakdown product of BSA marked *. The autoradiogram of the gel shows ADP-ribosylated proteins. Lanes 1 and 2, substrates incubated with [3H]NAD in the absence of PfSir2. Lane 1, histones; Lane 2, histones plus BSA. Lanes 3 to 6, substrates incubated with [3H]NAD in the presence of PfSir2. Lane 3, histones; lane 4, histones plus BSA; lane 5, histones plus BSA plus 10 mM nicotinamide (NAM); lane 6, PfSir2 alone. (B) Histones and BSA were treated with either PfSir2 or GST-tagged Hst2, as for panel A. The Coomassie blue-stained SDS-polyacrylamide gel shows the positions of PfSir2, histones, BSA, and GST-Hst2. Lane 3 is included to show the size of GST-Hst2, which is very close to the size of BSA. The autoradiogram of the gel shows ADP-ribosylation by PfSir2 but not by GST-Hst2. (C) After incubation of PfSir2 with histones and [3H]NAD as described for panel A, 5 μg phosphodiesterase (PDE) was added to half of the reaction mixture and incubated for a further 30 min at 37°C before TCA precipitation. Radiolabel on the treated protein was calculated as a percentage of that on the nontreated protein, normalized to the amount of Coomassie blue-stained Sir2 in each lane. (D) Coomassie blue-stained SDS-polyacrylamide gel with extensive resolution, showing PfSir2 incubated for 2 h with PDE or with buffer alone (−) and then repurified on Ni-NTA. a, original upper band; b, PDE-modified upper band; c, original lower band, unaltered by PDE. (E) In vitro deacetylation assays with substrate A were carried out in duplicate using PfSir2 treated as described for panel D. Activity is expressed as a percentage of the fluorescence generated by untreated PfSir2. The error bar indicates standard deviation.

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Polymerase. We have found no obvious modification occurs via sirtuins and what via poly-ADP-ribose and it remains unclear what proportion of functional histonelogenetic classes and are not closely homologous. Especially since TbSIR2RP1 and PfSir2 fall into different phylogenetic way (22), and it is interesting to speculate that this is related with a severe reduction in deacetylase activity. If PfSir2 did supply a genuine HDAC activity, this may be undirected since the DNA elements and/or protein binding partners that would normally recruit SpSir2 to pericentromeric DNA are unlikely to be closely conserved between the two species. Alternatively, PfSir2 may recognize, via relatively conserved sirtuin domains, the repetitive DNA structure of the S. pombe centromere. P. falciparum itself has extensive repetitive DNA at its telomeres, which are heterochromatic (19). In support of this second theory, S. pombe can be complemented by ScSir2 as well as PfSir2 (18), despite the absence of S. pombe homologs for the ScSir2 protein partners Sir3 and Sir4 and despite the fact that ScSir2 does not normally silence pericentromeric DNA in its own genome.

PfSir2 was unusual among other characterized sirtuins in having robust ADP-ribosyltransferase activity as well as deacetylase activity, as shown schematically in Fig. 7. Only TbSIR2RP1 has previously been reported to act in this bifunctional way (22), and it is interesting to speculate that this is related with the relative paucity of sirtuins in both parasites, especially since TbSIR2RP1 and PfSir2 fall into different phylogenetic classes and are not closely homologous.

The ADP-ribosylation of histones is a well-established phenomenon in mammalian cells, where it is associated with replication (8) and with the response to DNA damage (1, 6, 7, 37). This has not, however, been traced directly to sirtuin activity, and it remains unclear what proportion of functional histone modification occurs via sirtuins and what via poly-ADP-ribose polymerase. We have found no obvious P. falciparum homolog of poly-ADP-ribose polymerase or of its counterpart poly-ADP-ribose glycohydrolase, so if a DNA damage response involving ADP-ribose does occur in this species, it might be mediated by ADP-ribosylation via PfSir2. TbSIR2RP1 certainly seems to be involved in DNA damage survival in T. brucei (22). Alternatively, or perhaps additionally, mono-ADP-ribosylation of histones could act like acetylation or methylation as a mark used to control chromatin structure, either for transcriptional control or to facilitate DNA repair. However, histones in mammalian cells can be modified on a range of different residues, and sirtuins would be limited to modifying lysine unless they can ultimately transfer ADP-ribose to another acceptor residue within a target protein (Fig. 7, scheme 2). No mechanism for such a reaction has been published, but the concept arises in a recent report showing that HsSIRT4 regulates mitochondrial glutamate dehydrogenase (27), which is regulated by cysteine-specific rather than lysine-specific ADP-ribosylation (32). If this is so, PfSir2 could potentially modify a range of acceptor residues within its target histones, widening its scope beyond the deacetylation of lysine.

The fact that ADP-ribosylation was detected on PfSir2 itself as well as on its substrates may be due to the trapping of a stable reaction intermediate in which ADP-ribose is bound to the target protein. PfSir2 could potentially modify a range of acceptor residues within its target histones, widening its scope beyond the deacetylation of lysine. The concept arises in a recent report showing that HsSIRT4 regulates mitochondrial glutamate dehydrogenase (27), which is regulated by cysteine-specific rather than lysine-specific ADP-ribosylation (32). If this is so, PfSir2 could potentially modify a range of acceptor residues within its target histones, widening its scope beyond the deacetylation of lysine.

Turning to the possibility of using inhibitors or activators to modify PfSir2 activity, all of the most commonly used compounds that affect class I sirtuins were either ineffective or only minimally effective on PfSir2, possibly due to the lack of conservation between PfSir2 and the S. cerevisiae sirtuin from which these drugs were first selected (3). The exception was nicotinamide, a universal product of the NAD⁺-dependent deacetylase reaction which can act as a competing nucleophile attack-

![FIG. 7. Model of reaction schemes showing possible ADP-ribosylation and deacetylation outcomes of the sirtuin reaction. Scheme 1 shows the classical deacetylation reaction, producing O-acetyl-ADP-ribose and a deacetylated lysine in the target protein. Scheme 2 shows an ADP-ribosylation reaction in which ADP-ribose is either left attached to the target lysine or subsequently transferred to another residue within the target protein. Scheme 3 shows the transfer of ADP-ribose to the sirtuin instead of to the target protein.](image-url)
ing the peptidyl-ADP-ribose reaction intermediate (53). The deacetylation of histones by PfSir2 could accordingly be completely inhibited by sufficiently high levels of nicotinamide and at least partially inhibited within the K_range of the previously studied class I enzymes ScSir2 and Hst2 (110 μM and 180 μM, respectively) (50, 52). PfSir2 was much less responsive to nicotinamide when acting on a synthetic substrate, suggesting that the enzyme’s configuration may be altered when it binds to an actual physiological substrate. This is not without precedent, as the sensitivity of ScSir2 to nicotinamide is also altered by its binding to two different protein complexes in vivo (57). Interestingly, nicotinic acid (metabolized to nicotinamide in an in vivo mouse model) has been shown to directly modulate the expression of virulence genes in a sirtuin-dependent manner in the fungal pathogen Candida glabrata (13). This raises the possibility of targeting PfSir2 as a means of altering virulence gene expression and pathogenesis in P. falciparum.

In conclusion, this study characterizes PfSir2, which is one of only two sirtuins in the most important human malarial parasite, P. falciparum. PfSir2 is a genuine HDAC with all of the properties required to act as a controller of the epigenetic silencing of subtelomeric virulence genes. It is also an ADP-ribosyltransferase, an activity that certainly merits further investigation, as it may reveal both regulatory mechanisms and secondary functions for this sirtuin that are either related or unrelated to epigenetic silencing. Little is currently known about sirtuins in phylogenetic class III, and further work may now clarify the precise targets of PfSir2 in vivo, the mechanisms of its regulation, and the identity of any potential binding partners, leading to a better understanding of class III sirtuins in general and of epigenetic silencing in P. falciparum in particular.

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REFERENCES

1. Adamietz, P., and A. Rudolph. 1984. ADP-ribosylation of nuclear proteins in vivo. Identification of histone H2B as a major acceptor for mono- and poly(ADP-ribose) in dimethyl sulfate-treated hepatotha AH 7974 cells. J. Biol. Chem. 259:6841–6846.
2. Avalos, J. L., J. D. Boeke, and C. Wolberger. 2004. Structural basis for the mechanism and regulation of Sir2 enzymes. Mol. Cell 13:639–648.
3. Bedalov, A., T. Gathobton, W. P. Irvine, D. E. Gottschling, and J. A. Simon. 2001. Identification of a small molecular inhibitor of Sir2p. Proc. Natl. Acad. Sci. USA 98:15113–15118.
4. Berriman, M., E. Ghedin, C. Hertz-Fowler, G. Blandin, H. Renaud, D. C. Bartholomew, N. J. Lennard, E. Caler, N. E. Hamlin, B. Haas, U. Bohme, L. Hannick, M. A. Aslett, J. Shollam, L. Marcelli, L. Hou, B. Wickstead, U. C. Alsmark, C. Arrowhead, A. Banks, D. Johnson, D. Johnson, K. Jones, A. X. Kherkhounou, H. Koo, N. Larke, S. Landgraf, J. Larkin, V. Leech, A. Line, A. Lord, A. Macleod, P. J. Mooney, S. Moule, D. M. Martin, G. W. Morgan, K. Mungal, H. Norbertczak, D. Ormond, G. Pai, C. P. Seago, J. Peterson, M. A. Quail, E. Rabkinostrovich, M. A. Rajandream, C. Reiter, S. R. Salzberg, M. Sanders, S. Schoebel, S. Sharp, M. Simmonds, A. J. Simpson, L. Talton, C. M. Turner, A. Tait, A. R. Tivey, S. Van Aken, D. Walker, D. Wanless, S. Wang, B. White, O. White, S. Whitehead, J. Woodward, J. Wortman, M. D. Adams, T. M. Embley, K. Gull, E. Ullo, J. D. Barry, A. H. Fairlamb, F. Opperoedde, B. L. Barrett, J. E. Donelson, N. Hall, C. M. Fraser, S. E. Melville, and N. M. El-Sayed. 2005. The genome of the African trypanosome Trypanosoma brucei. Science 309:416–422.
5. Bitton, K. J., R. S. Anderson, H. Y. Coten, M. Latore-Esteves, and D. A. Sinclair. 2002. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J. Biol. Chem. 277:45099–45107.
6. Boulikas, T. 1988. At least 60 ADP-ribosylated variant histones are present in nuclei from dimethylsulfate-treated and untreated cells. EMBO J. 7:57–67.
7. Boulikas, T. 1989. DNA strand breaks alter histone ADP-ribosylation. Proc. Natl. Acad. Sci. USA 86:9976–9980.
8. Boulikas, T. 1990. Poly(ADP-ribosylated) histones in chromatin replication. J. Biol. Chem. 265:14638–14647.
9. Braunstein, M., R. E. Sobel, C. D. Allis, B. M. Turner, and J. R. Brouch. 1996. Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. Mol. Cell. Biol. 16:4349–4356.
10. Choo, K. J., R. Dzikowski, M. Frank, F. Li, A. Z. Jiwhi, D. L. Hartl, and K. W. Deitsch. 2007. Epigenetic memory at malaria virulence genes. Proc. Natl. Acad. Sci. USA 104:899–902.
11. Craig, A., and A. Scherf. 2001. Molecules on the surface of the Plasmodium falciparum infected erythrocyte and their role in malaria pathogenesis and immune evasion. Mol. Biochem. Parasitol. 115:113–122.
12. Dahmen, F., F. Martin, S. Perrey, and S. M. Gasser. 2006. A homotrimeric-heterotrimer switch in Sir2 structure differentiates rRNA and telomeric silencing. Mol. Cell. 21:825–836.
13. Domercq, R., C. Castano, A. De Las Penas, M. Zupancic, V. Lockelait, J. R. Helms, D. Johnson, and A. Ivens. 2005. Nicotinic acid limitation regulates silencing of Candida adhesins during U1. Science 308:866–870.
14. Duraisingh, M. T., T. Triglia, S. A. Ralph, J. C. Rayner, J. W. Barnwell, G. I. Simpson, L. Tallon, C. M. Turner, A. Tait, A. R. Tivey, S. Van Aken, D. W. Hall, and D. A. Sinclair. 2001. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. J. Biol. Chem. 276:27393–27398.
15. Frye, R. A. 1999. Characterization of five human cDNAs with homology to the yeast Sir2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. Biochem. Biophys. Res. Commun. 260:273–279.
16. Frye, R. A. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochem. Biophys. Res. Commun. 273:793–798.
17. Garcia-Salcedo, J. A., P. Gijon, D. P. Nolan, T. E. Pehabi, and E. Pays. 2003. A chromosomal Sir2 homologue with both histone NAD-dependent ADP-ribosyltransferase and deacetylase activities is involved in DNA repair in Trypanosoma brucei. EMBO J. 22:5851–5862.
18. Gasser, S. M., and M. M. Cockell. 2001. The molecular biology of the Sir2 proteins. Gene 279:1–16.
19. Gishelli, S., D. Donze, N. Dhillon, and R. T. Kamakaka. 2001. Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. EMBO J. 20:4522–4535.
20. Grozinger, C. M., E. D. Chao, H. E. Blackwell, D. Moazed, and S. L. Schreiber. 2001. Identification of a class of small molecule inhibitors of the yeast sir2 homolog(s) that deacetylates proteins in situ by a distinct class of deacetylase. J. Biol. Chem. 276:38837–38843.
21. Guarente, L. 2005. Calorie restriction and Sir2 genes—towards a mechanism. Mech. Ageing Dev. 126:923–928.
22. Haigis, M. C., R. Mostoslavsky, C. E. Haigis, K. Fahey, D. C. Christodoulou, A. J. Murphy, D. M. Valenzuela, G. D. Yancopoulos, M. Karow, G. Blander, C. Wolberger, T. A. Prolla, R. Windruch, F. W. Alt, and L. Guarente. 2006. Sir2 inhibition stimulates glutamate dehydrogenase and opposes the effects of calorie restriction in Drosophila. Cell 124:941–954.
23. Hallows, W. C., S. Lee, and J. M. Denu. 2006. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. Proc. Natl. Acad. Sci. USA 103:10230–10235.
29. Hecht, A., T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, and M. Grunstein. 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. Cell 80:383–393.

30. Heys, S. A., F. Dequidt, E. Verdin, and M. Jung. 2003. Nonisotopic substrate for assaying both human zinc and NAD+-dependent histone deacetylases. Anal. Biochem. 319:42–48.

31. Heltweg, B., J. Trapp, and M. Jung. 2005. In vitro assays for the determination of histone deacetylase activity. Methods 36:332–337.

32. Herrera-Voyer, A., S. M. Bakht, P. Franke, C. Weise, M. Schweiger, D. Jorche, and M. Ziegler. 2001. Regulation of glutamate dehydrogenase by reversible ADP-ribosylation in mitochondria. EMBO J. 20:2404–2412.

33. Howitz, K. T., K. J. Bitterman, H. V. Cohen, D. W. Lamming, S. Lava, J. G. Wood, R. E. Zipkin, P. Chung, A. Kiselevska, L. L. Zhang, B. Scherer, and D. A. Sinclair. 2003. Small molecule activators of Sir2 proteins extend Saccharomyces cerevisiae lifespan. Nature 425:191–196.

34. Hnai, S., C. M. Armstrong, M. Kaebeltein, and L. Guarente. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 403:795–800.

35. Kaebeltein, M., T. McDonagh, B. Heltweg, J. Hixon, E. A. Westman, S. D. Caldwell, N. A. Nishiyama, D. N. DiSferraro, S. Fields, A. Bedalov, and B. K. Kennedy. 2005. Substrate-specific activation of sir genes by reversionar. J. Biol. Chem. 280:17038–17045.

36. Khan, A. N., and P. N. Lewis. 2006. Use of substrate analogs and mutagenesis to study substrate binding and catalysis in the Sir2 family of NAD-dependent protein deacetylases. J. Biol. Chem. 281:11702–11711.

37. Kreimeyer, A., K. Wielckens, P. Adamietz, and H. Hilz. 1984. DNA repair-associated ADP-ribosylation in vivo. Modification of histone H1 differs from that of the principal acceptor proteins. J. Biol. Chem. 259:890–896.

38. Kyes, S. A., J. A. Rowe, N. Kriek, and C. I. Newbold. 1993. Thiamine-repressible expression vectors pREP and pRS425: evolutionarily conserved and nonconserved cellular localizations and functions of Sir2/Sir4 complex and evidence for a regulatory domain in Sir4 that inhibits its interaction with Sir3. Proc. Natl. Acad. Sci. USA 90:2186–2191.

39. Kaeberlein, M., T. McDonagh, B. Heltweg, J. Hixon, E. A. Westman, S. D. Caldwell, N. A. Nishiyama, D. N. DiSferraro, S. Fields, A. Bedalov, and B. K. Kennedy. 2005. Substrate-specific activation of sir genes by reversionar. J. Biol. Chem. 280:17038–17045.

40. Landry, J., A. Sutton, S. T. Tafrov, R. C. Heller, J. Stebbins, L. Pillus, and K. J. Pouvelle, J. Gysin, and M. Lanzer. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. EMBO J. 17:5418–5426.

41. Shankaranarayana, G. D., M. R. Motamedi, D. Moazed, and S. I. Grewal. 2003. Sir2 regulates histone H3 lysine 9 methylation and heterochromatin assembly in fission yeast.Curr. Biol. 13:1280–1286.

42. Tanny, J. C., G. J. Dowd, J. Huang, H. Hilz, and D. Moazed. 1999. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. Cell 99:735–745.

43. Trager, W., and J. B. Jenson. 1978. Cultivation of malarial parasites. Nature 273:621–622.

44. Tulin, A., N. M. Naumova, A. K. Menon, and A. C. Spradling. 2006. Drosophila poly(ADP-ribose) glycohydrolase mediates chromatin structure and Sir2-dependent silencing. Genetics. 172:363–371.

45. Vaquero, A., M. Scher, D. Lee, H. Erdjument-Bromage, P. Tempst, and D. Reinberg. 2004. Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. Mol. Cell 16:93–105.

46. Vaquero, A., M. B. Scher, D. H. Lee, A. Sutton, H. L. Cheng, F. W. Alt, L. Serrano, R. Sternlangua, and D. Reinberg. 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. Genes Dev. 20:1256–1261.

47. Voss, T. S., T. Mini, P. Jenoe, and H. P. Beck. 2002. Plasmodium falciparum possesses a cell cycle-regulated short type replication protein A large subunit encoded by an unusual transcript. J. Biol. Chem. 277:17493–17501.

48. Wilson, J. M., V. Q. Le, C. Zimmerman, R. Marmorstein, and L. Pillus. 2006. Nuclear export modulates the cytoplasmic Sir2 homologue Hst2. Mol. Cell. Biol. 26:6931–6946.

49. Saavedra, A., A. A. Xia, V. L. Schramm. 2003. Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. Biochemistry 42:9249–9256.

50. Scherf, A., R. Hernandez-Rivas, P. Buffet, E. Botiss, C. Benatar, B. Pouvelle, J. Gysin, and M. Lanzer. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. EMBO J. 17:5418–5426.