Identification of a novel post-translational modification in *Plasmodium falciparum*; protein sumoylation in different cellular compartments

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Summary

SUMO (Small Ubiquitin-like MOdifier) conjugation is a post-translational modification implicated in a variety of cellular functions including transcriptional regulation, nuclear location and signal transduction. Sumoylation, although conserved and vital in eukaryotes, has not been studied in malaria parasites. Here, we identify SUMO conjugation of blood stage parasites of *Plasmodium falciparum*. Antibodies raised against synthetic peptides of the plasmodial SUMO orthologue PfSUMO, a 100-amino-acid protein, reacted with distinctive subcellular compartments of the parasitized erythrocyte during blood stage development. Anti-PfSUMO stains the nucleus and parasite cytoplasm. We also found antibody reactivity in the host cell cytoplasm with the parasite-derived structures called Maurer’s clefts. Anti-PfSUMO reacts in Western blot with a number of blood stage proteins ranging from approximately 40–250 kDa. Parasites expressing FLAG-tagged PfSUMO gave similar results in Immunofluorescence assay and Western blots. In addition, we show that anti-PfSUMO identified PfSir2, a telomere-associated nuclear protein involved in var gene silencing, as a target for sumoylation. Furthermore, LC-MS/MS analysis of a two-step immunoprecipitation (IP) with anti-FLAG and anti-PfSUMO antibodies reveals a number of putative *P. falciparum* sumoylated proteins. Our results imply that SUMO conjugation has an essential function in a number of different biological processes in *P. falciparum*.

Introduction

Gene expression in eukaryotes is executed by a relatively small number of regulators, the dynamic prospects of which are dramatically increased by post-translational modifications. Reversible post-translational modifications of proteins, such as phosphorylation, acetylation, methylation and ubiquitination have critical roles in many cellular processes owing to their ability to cause rapid changes in functions of pre-existing proteins and subcellular structures. Covalent attachment of one protein to another, resulting in altered localization and/or function, is one of the best-studied post-translational modifications with respect to prevalence in eukaryotes. Among these, ubiquitination is the most studied; however, other ubiquitin-like proteins (Ubls), such as SUMO, Nedd 8 and ISGN15, are gaining increased attention (Jentsch and Pyrowolakis, 2000).

*Plasmodium falciparum*, the most virulent of the protozoan parasites infecting humans, causes a medically and socio-economically debilitating form of malaria. Although components of the ubiquitination pathway and related ubiquitin-like modification, such as neddylation has recently reported in malaria parasites (Artavanis-Tsakonas et al., 2006), protein sumoylation in apicomplexan parasites has not been investigated. This, despite the fact that SUMO is emerging as a flexible modifier for a large number of proteins in many different pathways with diverse consequences and targets (Seeler and Dejean, 2003). SUMO is structurally related to ubiquitin (Bayer et al., 1998), is of similar size and is also ligated to lysine residues within its target proteins. This happens via a cascade of activation, conjugation and ligation steps, to produce an isopeptide bond between C-terminal glycine of SUMO and a specific lysine (within a short consensus sumoylation motif) of the target protein (Johnson, 2004). However, unlike ubiquitin it does not mark the protein for degradation, instead it modifies its substrate’s interaction with partner proteins. In contrast to the single SUMO found in Saccharomyces cerevisiae, Drosophila melanogaster and Caenorhabditis elegans, higher eukaryotes express multiple SUMOs, with up to eight members in Arabidopsis thaliana and three in humans, namely SUMO1, SUMO2 and SUMO3. Mature SUMO2 and

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SUMO3 are nearly identical (~95% identity) but differ substantially from SUMO1 (~50% identity).

The complex life cycle of the human malaria parasite *P. falciparum*, divided between insect vector and human host, necessitates dynamic protein expression and gene regulation to exploit efficaciously the different host environments. Clearly the parasite requires a dynamic protein repertoire and a ubiquitous control of gene expression to achieve such complex tasks. This has been generally evidenced by reports of considerable levels of gene expression regulation throughout the *P. falciparum* life cycle (Florens *et al.*, 2002; Lasonder *et al.*, 2002). Furthermore, the scarcity of plasmodial transcription regulators identified (Coulson *et al.*, 2004) indicates alternative modes of control. A good example of a prominent role of reversible post-translational modifications in gene regulation comes from the study of histone marks and the telomeric chromatin-associated PISir2 protein in antigenic variation of *P. falciparum* (Freitas-Junior *et al.*, 2005; Chookajorn *et al.*, 2007; Cui *et al.*, 2007; Lopez-Rubio *et al.*, 2007). Here we demonstrate the presence of another post-translational modification in malaria parasites called sumoylation. We report data that imply a crucial role of SUMO-conjugated proteins in different processes during *P. falciparum* blood stage development.

**Results**

**Identification of *P. falciparum* SUMO1/SMT3 homologue and sumoylation pathway components**

Growing evidence of the presence and role of ubiquitin-like modifiers in gene regulation in model eukaryotic systems led us to search for putative Ubls in *P. falciparum*. Amino acid sequence comparisons revealed a putative Ubl catalogued with gene ID PFE0285c (previously MAL5P1.58) in PlasmoDB as having the best score. Based on the best-hit score we have hereby called it PISUMO. Multiple alignments with other previously characterized SUMO orthologues show that PISUMO is 46% identical to *S. cerevisiae* SMT3, 40% identical to Human SUMO1, Mouse SMT3 and *S. pombe* Pmt3 and 47% and 43% to Human SUMO2 and 3 respectively (Fig. 1A). To exclude the possibility of obtaining a protein involved in ubiquitination or some other Ubls instead, we carried out similar alignment(s) with known yeast and human ubiquitin, revealing only about 11% and 7% identity, respectively, to PISUMO (Fig. S1). Furthermore, PISUMO lacks the critical Lys48 and Lys63 residue of ubiquitin, required for the generation of ubiquitin polymers and instead seems to possess the most prominent feature of SUMO1, a long N-terminus, which is believed to be highly flexible and protruding from the core of the protein (Bayer *et al.*, 1998). However, the two C-terminal Gly residues required for isopeptide bond formation are conserved between all sequences. These bioinformatic analyses and observations together suggest the protein to be of the SUMO family. Interestingly, though it shares slightly more identity to SUMO2, other features such as high similarity score with *S. cerevisiae* SMT3 and apparent structural features such as the long N-terminal point it to be more similar to SUMO1.

The complete characterization of a protein involved in a crucial process necessitates deciphering and establishing its entire pathway. We thus carried out homology search for enzymes involved in the SUMO pathway for yeast and humans against the *P. falciparum* database. *P. falciparum* orthologues of all members of the pathway have been identified and the proposed pathway components are represented (Fig. 1B). Like its most widely known counterpart SMT3, SUMO1 is conjugated to target proteins by a pathway that is distinct from, but analogous to, the ubiquitin conjugation. Similar to known SUMO E1 enzymes, which are distinct from ubiquitin-specific enzymes (Desterro *et al.*, 1997; Johnson and Blobel, 1997), *P. falciparum* E1 consists of a heterodimer of two enzymes, Uba2 and Aos1. The E2 homologue with conjugating activity is the Ubc9, which also acts as the SUMO ligase (Johnson and Blobel, 1997). The pathway is completed with the SUMO proteases, which have the dual function of de-sumoylating the target by cleaving the substrate bound SUMO peptide, as well as to process the mature SUMO by cleaving it to leave the diglycine motif at the terminus for isopeptide bond formation.

**Detection of putative sumoylated proteins in *P. falciparum***

Western blot analysis of *P. falciparum* total parasite extracts was performed, revealing several bands corresponding to potentially sumoylated plasmodial proteins (Fig. 2). Anti-PISUMO guinea pig sera stains a number of different proteins within the range of ~40 kDa to < 250 kDa in strain 3D7 parasite extracts. The pre-immune sera did not recognize the large majority of the proteins. Similar results were observed in extracts from transfectants bearing endogenously FLAG-tagged PISUMO (Fig. 2). Mouse anti-FLAG antibody stains an essentially comparable pattern in PISUMO-FLAG transfectants, although revealing a few more potentially sumoylated proteins, perhaps due to higher sensitivity resulting from tagging. In-depth analysis of the membrane and cytosolic proteome of red blood cell did not reveal any evidence of sumoylated proteins so far (Pasini *et al.*, 2006). Nevertheless, to exclude any host-specific sumoylated proteins and confirm specificity of the antibodies, extracts from uninfected red blood cells were also blotted with the above-mentioned antibodies (Fig. 2, right panel).
Extracts obtained at specific stages, i.e. the early ring stages, mature trophozoite and schizont stages, revealed differential pattern (data not shown), indicating that the modification targets stage-specific proteins or modifies the same protein differentially in a stage-specific manner.

To address the question as to where SUMO target proteins localize, we performed immunofluorescence assays.
(IFAs) on fixed, synchronized wild-type parasites at specific stages with anti-PSUMO antibodies (Fig. 3A). Staining was observed in all the blood stages with a differing pattern across the stages. Ring stage wild-type 3D7 parasites showed partial nuclear staining along with around 5–6 Maurer's clefts-like vesicular structures in the host cytoplasm. A similar pattern was observed during trophozoite stages but the number of vesicular structures increased (Fig. 3A, middle panel). At schizogony the picture became more dense as diffused staining, mainly throughout the nuclei, was observed (Fig. 3A, right panel). SUMO modification in other systems is known to target mainly nuclear proteins, although many reports implicate SUMO in modifying cytosolic proteins specifically targeting nuclear-cytosolic export. Furthermore, the pattern appeared to be similar to the P. falciparum Maurer's clefts, vesicle-like structures that are involved in the trafficking of PfEMP1 and other virulence proteins to the host cell surface. IFAs on PSUMO-FLAG transfectants yielded similar staining pattern throughout the life cycle when probed with anti-FLAG antibody, with the exception of the Maurer’s clefts-like pattern not being detectable in most parasites analysed (Fig. 3B). A minority (approximately 1%) of the PSUMO-FLAG-infected parasites, when assayed with anti-FLAG antibodies did, however, show the host cytosolic staining (Fig. 3C). This may be explained by altered characteristics of the tagged PSUMO.

However, intrigued by the Maurer's clefts-like staining by PSUMO sera, we undertook colocalization IFAs with P. falciparum markers staining specific parasite compartments and anti-PSUMO antibodies (Fig. 4). To this end, we used anti-PfSir2 (Freitas-Junior et al., 2005), a nuclear marker, and observed partial colocalization of PSUMO with the peripheral nuclear staining pattern of PfSir2. This demonstrated the nuclear staining by anti-PSUMO and is consistent with a possible association with PfSir2 (Fig. 4A, top panel). Next we used anti-PfHsp70-1 (Mattei et al., 1989) as a cytoplasmic marker and observed a colocalization with PSUMO (Fig. 4A, middle panel). To further investigate the observed extra parasitic host cytosolic staining, we used a marker that stains the P. falciparum Maurer's clefts, mAb51-22 (Hinterberg et al., 1994). We observed complete colocalization of the
PfSUMO vesicular dots with Maurer’s clefts (Fig. 4A, bottom panel). These assays documented the presence of putatively sumoylated proteins in different parasitic and extra parasitic compartments (Fig. 4B).

To test for specificity of the PfSUMO serum antibodies, we performed peptide competition assays and subsequent immunofluorescence. The specific staining pattern in the parasite and host cell could be inhibited in a dose-dependent manner conferring that the IF staining is specific (Fig. S2). IFAs were also carried out with non-fixed, air-dried parasites and identical results obtained (data not shown).

Fig. 3. PfSUMO immunofluorescence assays throughout the blood stage cycle.
A. Reactivity of anti-PfSUMO sera on wild-type 3D7 strain parasites.
B. Reactivity of anti-FLAG (tag) antibody on PfSUMO-FLAG-tagged parasites. Ring stage 10–14 h, trophozoite stage 26–30 h, schizont stage 40–46 h post invasion.
C. Comparison of IFA pattern obtained by anti-PfSUMO and anti-FLAG antibodies on PfSUMO-FLAG-tagged parasites (trophozoites). Size bar corresponds to 1 μm. Nuclear staining by DAPI in blue.
Identification of PfSir2 as a SUMO substrate

PfSir2 is a nuclear plasmodial protein that is implicated in control of var gene transcription (Duraisingh et al., 2005; Freitas-Junior et al., 2005). During characterization of this protein we observed a discrepancy from the predicted molecular weight of 30 kDa and instead observed a major band of approximately 72 kDa on immunoblot-
We questioned whether this shift in size could be due to a post-translational modification like sumoylation. On scanning the PfSir2 for probable sumoylation motifs (Rodriguez et al., 2001), a consensus sequence of four amino acids, most widely validated as SUMO binding motif (yKx E/D), we found three motifs, one with high probability and two with low probability that were potential SUMO binding sites (http://www.abgent.com/doc/sumoplot, Fig. 5A). Following this we carried out immunoprecipitation (IP) assays with anti-PfSir2 and anti-PfSir2 antibodies and probed the precipitated fractions with anti-PfSUMO and anti-PfSir2 sera respectively (Fig. 5B and C). The fraction immunoprecipitated with anti-PfSUMO (Fig. 5B) recognized a faint yet distinct band at around 72 kDa on being probed with anti-PfSir2 along with a fainter fraction at around 60 kDa. The same fraction when blotted with PfSUMO as positive controls (first two lanes), anti-PfSUMO (third lane) and anti-IgG (fourth lane). Arrow marks corresponding PfSir2 band(s) observed in anti-PfSUMO immunoprecipitated protein extract.

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Identification of sumoylated candidate proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS (LTQ))

Sumoylation is a global modification with widespread targets and far-reaching effects. We were interested in generating a first list of putative SUMO targets in *P. falciparum* to gain insight into the cellular processes affected by this modification. Taking advantage of the FLAG-tagged PISUMO strain, we conducted a two-step purification of SUMO target proteins. IPs were carried out, first with monoclonal anti-FLAG antibody followed by anti-PfSUMO sera, thus increasing the stringency of the purification. Accurate mass (MS) and sequence information (MS/MS) was obtained by standard in-gel trypsin digestion of bands specific only to PISUMO sera and treatment of resulting eluates in the mass spectrometer. Information acquired was used to identify the peptides against the *Plasmodium* database using SEQUEST algorithm. A list of corresponding proteins was generated based on the analysis (Table 1). Although a number of hits were obtained using stringent filtering criteria, only a fraction was identified by two or more peptides generating more confidence than those identified by single peptides. This is not surprising, considering the reported low abundance of sumoylated proteins at a given time in analogous systems. It is noteworthy though that among all the substrates recognized, a good number had orthologues known to be sumoylated individually and/or as part of a complex in other model systems (marked by asterisk '*' in Table 1). All the identified proteins harbour potential sumoylation motifs (data not shown), though due to the limitation of the mass spec technique, the exact residue(s) could not be identified.

**Discussion**

In this work we present, for the first time, experimental evidence that *P. falciparum* utilizes SUMO conjugation to modify blood stage proteins. Antibodies raised against a *P. falciparum* SUMO peptide orthologue as well as against

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**Table 1.** Putative SUMO substrates identified by LC-MS/MS analysis of *Plasmodium falciparum* proteins.

| Gene ID^a^ | Protein name                                      | Putative function | Location | No. of peptides |
|-----------|---------------------------------------------------|-------------------|----------|-----------------|
| Proteins identified as sumoylation substrates by two or more peptides |                     |                  |           |                 |
| PF0415c   | Hypothetical Protein                              | ND                | ND       | 6               |
| PFC0140c  | N-ethylmaleimide-sensitive fusion protein, putative | Vesicular trafficking | IE       | 6               |
| PF11.0061*| Histone H4                                        | Inversion         | Merozoite| 5               |
| PF14.0334 | NAD(P)H-dependent glutamate synthase, putative    | ND                | Glutamate metabolism | 2               |
| PF14.0713c| Hypothetical Protein                              | ND                | ND       | 2               |
| PF07.0030 | Heat shock protein 86 family                      | Heat shock protein | ND       | 2               |
| PF10.0161 | PHIST domain protein                              | Chromatin structure | ND | 2               |
| Proteins identified as sumoylation substrates by single peptide |                     |                  |           |                 |
| PF11.0381 | Subtilisin-like protease2                         | Inversion         | Micronemes|                 |
| PF10.0055 | Hypothetical Protein                              | ND                | ND       |                 |
| PF11.0404*| Transcription factor with AP2 domain(s)           | ND                | ND       |                 |
| PF14.0067 | LCL domain-containing protein CCP3                | ND                | ND       |                 |
| PF14.0713 | RNA-binding protein/splicing factor               | ND                | ND       |                 |
| MAL7P1.68 | Zinc finger containing ERF2 subunit of a palmitoyltransferase | Palmitoylation | ND       |                 |
| MAL8P1.7  | DNA-directed RNA polymerase II second largest subunit, putative | Transcription | ND       |                 |
| PF0975c   | Conserved plasmidium protein                      | ND                | ND       |                 |
| PFF1440v* | SETdomain protein                                 | Chromatin structure | ND |                 |
| PF10.0368*| Dynamin protein                                   | Trafficking/organelle fission/ | Cytoplasm, ER-Golgi, Apicoplast | |

^a. [http://plasmodb.org](http://plasmodb.org)

*Orthologues known to be directly sumoylated and/or part of sumoylated complex.
ND, not determined; IE, infected erythrocyte.
the epitope used to tag the PfSUMO detect, by Western blot, more than a dozen parasite proteins expressed during the intracellular blood stage cycle. Many proteins seen in total parasite extracts are also detected in nuclear extracts (Figs 2A and 5A), indicating that the majority of sumoylated proteins reside in the nucleus. Unlike ubiquitination, SUMO conjugated proteins are not marked for degradation, but rather have a number of eclectic functions in eukaryotic organisms such as in protein–DNA interactions (Goodson et al., 2001; Hong et al., 2001), regulation of transcription factor and chromatin-remodelling protein(s) (Gill, 2003) among many other biological processes. A knowledge of the proteins targeted by this modification is thus of utmost importance in deciphering its impact on the biology of the organism. To this end, we have also generated the first list of candidate-sumoylated proteins in plasmodium by mass spectrometry approach. Some of these are of particular interest for the parasite biology (Table 1) and may be pursued for further investigation. Given the limiting amount of plasmodial extracts obtainable as compared with other model organisms (i.e. yeast, mammalian) as also the low abundance of enriched SUMO conjugates present at a given time, identification of sumoylated targets is technically challenging and requires an upscaling of the initial starting material and more sensitive instrumentation.

The staining of anti-PfSUMO with Maurer’s clefts indicates that the parasite may traffic SUMO-conjugated proteins outside the conventional intracellular compartments into the erythrocyte cytoplasm (see Fig. 4B). The robust appearance of an NSF fusion protein in the mass spec analysis complements this observation (Table 1). This protein is known to appear as cytoplasmic vesicular structures with putative role in vesicular trafficking (Hayashi et al., 2001). Combining these observations, it is tempting to speculate that PfSUMO may take part in the regulation of virulence factor trafficking to the infected erythrocyte (IE) surface, as has been described for another post-translational modification, the phosphorylation of Maurer’s clefts proteins by parasite exported kinases (Droucheau et al., 2004; Nunes et al., 2007). Our efforts to identify sumoylated proteins in purified ghosts of IE failed using our anti-PfSUMO antibodies (data not shown). This may be due to the low abundance of proteins that carry this modification. In FLAG-tagged parasites, only a minor fraction of the IE cytoplasm was stained, indicating that the pathway leading to the Maurer’s clefts trafficking may be disturbed by the presence of the FLAG tag.

Having observed the unusual molecular weight of the nuclear protein PfSir2 in Western blot in nuclear extracts, we speculated that it may be a substrate for SUMO ligase(s). The open reading frame predicts a protein of approximately 30 kDa and the apparent MW is 72 kDa using a number of different anti-PfSir2 antibodies (Freitas-Junior et al., 2005). Given that three putative sumoylation sites are found in the PfSir2 sequence (Fig. 5A), full sumoylation would predict a molecule of approximately 70 kDa. Our data strongly support SUMO conjugation causing the increase in molecular weight of PfSir2. The fainter band at approximately 52 kDa (Fig. 5B) could be either a differentially spliced form or a degradation product. PfSir2 is known to be associated with the nuclear periphery with telomeres and nucleolus. We hypothesize that PfSir2 sumoylation determines the nuclear location of PfSir2. Mutant PfSir2 revealed in P. falciparum that this protein is involved in a number of different functions, such as gene silencing of a subgroup of telomere-adjacent var genes (Duraisingh et al., 2005; Freitas-Junior et al., 2005) and telomere length control (A. Scherf, unpubl. data). Work to investigate whether mutating the three putative SUMO conjugation sites may lead to differential subcellular location of PfSir2, or alter var gene de-repression and telomere length, is underway. The yeast Sir2 is not sumoylated (Denison et al., 2005). However, other HDACs have been shown to be sumoylated (David et al., 2002; Cheng et al., 2004) with altered biological activity in terms of levels of protein acetylation and/or gene repression. Interestingly, very recent studies show SIRT1, the closest mammalian homologue of yeast Sir2, to be sumoylated thereby increasing its deacetylation activity (Yang et al., 2007).

Histone sumoylation in S. cerevisiae has been recently observed, implicating it as an alternative mechanism akin to other chromatin modifications, such as methylation and acetylation (Nathan et al., 2006). We investigated whether P. falciparum histones carry SUMO marks. Histone H4 specifically came up as a SUMO substrate in our mass spec analysis (Table 1). Using semi-purified histones and reacting with anti-PfSUMO sera, we observed a faint band migrating higher than the predicted molecular weight of histones (this work, unpublished data). Also, this observation is compatible with a minor fraction of histones being conjugated to SUMO and argues for a possible role of sumoylation as a negative regulator in malaria parasites. Interestingly, the H2AZ homologue seemed to accumulate as many as six potential SUMO motifs in the N-terminal region (H2AZ – 6 sites – K11, K15, K25, K30, K38, K153; H2A – 4 sites K6, K21, K36 and K124), outnumbering those present in yeast H2AZ and H2A. Similar tandem array of potential sumoylation sites in the N-terminal region were also seen on other apicomplexans, e.g. the T. gondi H2AZ (with seven motifs in the N-terminal). Given the exposure of the N-terminal region of histones to enzymatic modifications, it points to a possible differential utilization of the same lysine residues for different modifications.

Stabilizing proteins by competing with ubiquitin (Desterro et al., 1998) and regulation of binding to
promoter DNA (Boyer-Guittaut et al., 2005) were the only mechanisms known to be associated with SUMO modification of transcription factors, strictly limiting it to target-SUMO covalent conjugation. Recently, though SUMO has been suggested to have a transcriptionally repressive role, mainly by promoting non-covalent interactions with co-repressors. Multitude of chromatin (histone) modifying enzymes, including deacetylases, methyltransferases and demethylases, are proposed to be among the many transcriptional regulators that interact non-covalently with SUMO to bring about repression (Yang et al., 2003; Rosendorff et al., 2006). In line with this, a number of putative sumoylated proteins identified in our mass spec study are potential transcription and chromatin regulators and orthologues of some of those identified are known SUMO targets (Table 1). The proteins detected in our analysis are probably only the most dominant ones and represent only ‘the tip of the iceberg’. Nonetheless, identification of SUMO – interacting domains on these and other potential candidates will be of utmost importance. Moreover, the physical association of SUMO with large multiprotein complexes like nuclear lamina proteins, PML nuclear bodies and centromeres may suggest SUMO in maintaining stability of multiprotein complexes (Zhong et al., 2000; Seeler et al., 2001), an interesting avenue to explore in a system like Plasmodium where multiprotein complexes are known to work in tandem for important processes like trafficking and silencing, etc.

In summary, despite the growing knowledge on the importance of this relatively novel modification, little is known about its precise biological impact/outcome even in extensively studied model systems, and none in apicomplexan before this study. The list of newly discovered SUMO substrates is expanding only recently in other model systems whereas none existed so far for plasmodium before this study. No unifying role could be so far associated with sumoylation in eukaryotes and it appears that each studied class of protein adds a new function to the long list of described biological roles (Seeler et al., 2007; Xhemalce et al., 2005). Our work on malaria parasites supports this notion, as we observe sumoylation of the telomere-associated protein PfSir2, involved in gene silencing, and we present evidence of a putative SUMO substrate that is exported from the parasite into the host cytoplasm via Maurer’s clefts. Both observations have not been previously associated with SUMO and support the notion in malaria parasites of ‘The usual suspect in a new scenario’. Obviously, a first logical step would be to do an in-depth and detailed proteomic profile of the same to identify more SUMO substrates in blood stage parasites of P. falciparum and identify their critical role in the biology of the parasite such as immune escape, invasion process and gene regulation. Both Plasmodium-specific anti-PfSUMO antibody and the FLAG-tagged SUMO strain generated in our work provide a valuable tool to explore the biological impact of this modification in the pathogen. The list of putative plasmodial SUMO substrates further provides direction to this understanding.

**Experimental procedures**

*P. falciparum cultures*

Plasmodium falciparum blood stage parasites from the 3D7 strain were cultured using modifications to the method described by Trager and Jensen (1976). Briefly, parasites were grown in O+ human erythrocytes in RPMI 1640 medium containing L-glutamine (Invitrogen) supplemented with 5% v/v human AB serum (PAA Laboratories GmbH) and 5% v/v Albumax II (Invitrogen) in a gas environment of 5% CO2, 1% O2 and 94% N2. Cultures were synchronized with two consecutive 5% sorbitol (Sigma) treatments (Lambros and Vanderberg, 1979). For kinetic IFAs the parasites were synchronized within a window of approximately 6 h, monitored by Giemsa staining.

**Bioinformatic analysis**

Genes coding for putative ubiquitin-like proteins in *P. falciparum* were obtained both from PlasmoDB (http://www.plasmodb.org) and GeneDB (http://www.genedb.org/genedb/malaria/). To identify the plasmodial SUMO gene (PfSUMO), previously characterized yeast SUMO/SMT3 orthologues (YDR510W) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and used as query on TBLASTN. Multiple sequence alignments were constructed using CLUSTALW algorithm using the BLOSUM series of similarity matrix with gap opening and extension penalties of 10 and 0.1 respectively.

**Antibodies**

To obtain polyclonal antibody serum against PFE0285c (PfSUMO1), we used the Eurogentec standard protocols (http://www.eurogentec.com) for immunizing guinea pigs and mice. Each animal was immunized with a combination of two synthetic peptides. The peptide sequences are as follows: H2N-[C]GGEHIQKVRSPDGA-CNH2 and H2N-[C]YGDRIHG DNTPEQL- The peptide sequences are as follows: H2N-[C]GGEHIQKVRSPDGA-CNH2 and H2N-[C]YGDRIHG DNTPEQL- BLAST search using individual peptide sequence(s) against the *P. falciparum* database did not yield any significant hits. Anti-FLAG mouse monoclonal antibody was obtained from Sigma. Co-localization assays were carried out with antibodies directed against specific markers: nuclear marker, PfSir2 (Freitas-Junior et al., 2005), parasite cytoplasmic marker, PfHsp70-1 (Mattei et al., 1989) and Maurer’s clefts marker, mAb51-22 (anti-Pf332) (Hinterberg et al., 1994).

**Plasmid construct and parasite transfection**

PfSUMO gene was amplified using Expand High Fidelity PCR System (Roche) with primers containing restriction sites for the enzymes XhoI and AflII, forward primer 5′-CCGCTCGAG ATGGGAGATGACGATTCAGC-3′, reverse primer 5′-AAAAA CTTAAGTTAGAAACTTCCCTCTTGTGGCTGAACC-3′ (enzyme
restriction sites in bold) and cloned in frame at the C terminal of 1x-FLAG tag previously cloned into the backbone plasmid vector pLN-ENR-GFP (Nkrumah et al., 2006). 3D7 ring stage parasites were transfected by electroporation and drug selected using bacterial integrase mediated transfection system as previously described (Nkrumah et al., 2006) to obtain chromosomal integration of the tagged construct.

Immunofluorescence microscopy

Synchronized IEs (ring stage 10–14 h, trophozoite stage 26–30 h, schizont stage 40–46 h) were washed in phosphate-buffered saline (PBS) (0.15 M NaCl and 10 mM sodium phosphate, pH 7.2). Cell pellets were re-suspended in 5 vols of PBS and a monolayer was set onto microscope slides. Parasites were air-dried and fixed for 10 min at room temperature in 4% paraformaldehyde. Slides were blocked for 20 min in 0.1% bovine serum albumin (BSA) and incubated with the primary antibody diluted in 0.1% BSA for 45 min at room temperature. After washing, cells were incubated for 30 min with a secondary antibody conjugated with appropriate fluorochrome (Alexa Fluor 488, Alexa Fluor 568, Molecular Probes) in a 1:500 dilution. Slides were washed thoroughly in PBS and mounted in Vectashield anti-fading containing 4-6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories). Images were captured using a Nikon Eclipse 80i optical microscope.

Anti-PfSUMO sera were pre-absorbed with lysed erythrocytes before incubation with the IEs. The final antibody dilutions were 1:200 for both PfSUMO sera and pre-immune, 1:500 for anti-FLAG, 1:100 for anti-PfSir2, 1:500 for anti-PfHsp70-1 and 1:400 for anti-mAb51-22 for colocalization assays.

Peptide competition assays were performed to test the specificity of the anti-PfSUMO sera. Increasing concentrations of the specific and unspecific peptides, respectively (ranging from 3 μg of respective peptide per ml to 3000 μg per ml), were added to the serum and incubated overnight in a rotating wheel at 4°C. The serum was subsequently used to carry out IFA as described above.

Western blot analysis

Equal amounts of parasite protein extracts from 3D7 strain and PfSUMO-FLAG transfected strain were separated by 4–12% SDS-PAGE on gradient gels (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were blocked at room temperature for 1 h in PBS, 0.05% Tween-20 and 5% non-fat milk and probed with antibody against PfSUMO and pre-immune sera overnight at 4°C. Secondary antibodies conjugated to horseradish peroxidase (Pierce) were incubated for 1 h at room temperature and developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions. Precision Plus Pre-stained molecular weight marker was obtained from Bio-Rad.

Immunoprecipitation assays

Briefly, ~4 × 10⁷ parasites (trophozoite stage 26–30 h post invasion) were isolated from IE by saponin lysis. Parasites were re-suspended in Lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.25% NP-40] containing a mixture of protease inhibitors (Roche) and set on ice for 30–60 min. Parasites were then lysed by 100–300 strokes in a homogenizer, the nuclei recovered by centrifugation, re-suspended in 100 ml of sonication buffer and then incubated on ice for 15 min followed by sonication with a Bioruptor Sonicator according to manufacturer’s instructions. After sonication-insoluble material was removed by centrifugation, lysates were pre-cleared by incubation with protein A sepharose CL-4B (Amer-sham) for 1 h. The extracts were incubated overnight at 4°C with 15 μl each of PfSUMO antisera and the pre-immune serum, anti-PfSir2 and anti-IgG. Immunoprecipitates were collected with protein A sepharose, and washed once each with low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl, protease inhibitors], High-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl, protease inhibitors] and LiCl-immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1], protease inhibitors) followed by a wash in TE buffer. The immunoprecipitated proteins were eluted from the sepharose beads by addition of Lamelli buffer and boiling for 10 min at 95°C. The eluates were separated on 4–12% SDS gels and blotted with appropriate antibodies as described above.

Mass spectrometry analysis

Two-step IPs were performed, as above, on extracts from asynchronous blood stage cultures of PfSUMO-FLAG transfected strain. Monoclonal anti-FLAG antibody was used in the first step IP and eluate was subjected to second IP with anti-PfSUMO and pre-immune sera respectively. After standard in-gel trypsin digestion, samples were subjected to MS/MS spectrometry using a LTQ ion trap mass spectrometer. The collection of resulting spectra was then searched against a plasmodium database (PlasmoDB) using the SEQUEST algorithm. Peptides identified were subjected to stringent search criteria with Xcorr values greater than 1.9, 2.2, 3.75 and 4.5 for 1, 2, 3 and 4 spectra, respectively, and Delta CN > 0.1.

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**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1.** PISUMO versus ubiquitin alignment. CLUSTAL W algorithm was used to ascertain PISUMO homology to known ubiquitin and Ubl orthologues. Multiple alignment between human ubiquitin and Ned8, *S. cerevisiae* Ubiquitin and Ned8-like RUB1 and *S. pombe* Ned8 was done. Dark shading represents residues identical in all sequences. Light shading, residues similar between respective sequences. Box, conserved Lys 48 and Lys 63, which serve as common sites of ubiquitination polymerization, which are missing in PISUMO sequence, as pointed with arrow(s).

**Fig. S2.** PISUMO peptide competition assay. IFA after competing PISUMO antisera with PISUMO peptide (A) and unspecific peptide (B). Horizontal grey bar above IFA panel depicts increasing amount of specific and unspecific peptide used, respectively, to compete the antisera (3–3000 μg ml⁻¹).

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