

Identification by functional proteomics of a deubiquitinating/deNeddylating enzyme in *Plasmodium falciparum*

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Summary

Ubiquitination is a post-translational modification implicated in a variety of cellular functions, including transcriptional regulation, protein degradation and membrane protein trafficking. Ubiquitin and the enzymes that act on it, although conserved and essential in eukaryotes, have not been well studied in parasites, despite sequencing of several parasite genomes. Several putative ubiquitin hydrolases have been identified in *Plasmodium falciparum* based on sequence homology alone, with no evidence of expression or function. Here we identify the first deubiquitinating enzyme in *P. falciparum*, PfUCH54, by its activity. We show that PfUCH54 also has deNeddylating activity, as assayed by a mammalian Nedd8-based probe. This activity is absent from mammalian homologues of PfUCH54. Given the importance of parasitic membrane protein trafficking as well as protein degradation in the virulence of this parasite, this family of enzymes may represent a target for pharmacological intervention with this disease.

Introduction

Malaria, the disease caused by *Plasmodium* parasites, is a widespread and debilitating illness of utmost public health importance (Snow *et al.*, 2005). Despite all efforts to control the morbidity and mortality associated with this parasite, a safe, efficacious vaccine has yet to be developed. Current preventive and curative drug regimens are often plagued by unpleasant side-effects and, more importantly, limited

effectiveness due to *Plasmodium*'s efficiency in developing resistance (Schellenberg *et al.*, 2006). Consequently, novel vaccine and drug targets are necessary for the successful control of malaria.

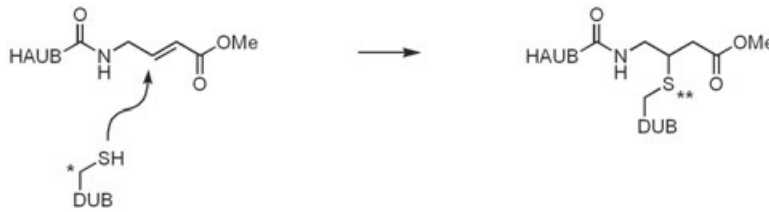
The key to a good therapy lies in the ability to specifically target a parasitic function with minimal effect on the host. The essential nature of ubiquitin (Ub) modifications suggests that the enzymes acting on it may be potential drug targets. Ub and some of its relatives, including Nedd8, SUMO and ISG15, are dynamic post-translational modifiers that can be added or removed from proteins to achieve a certain localization or function. Ub is the most extensively characterized and studied modifier, involved in a variety of cellular processes including protein trafficking, protein quality control, transcriptional regulation and signalling. Its addition to target proteins is mediated by E1 Ub activating enzyme, E2 Ub conjugating enzymes and E3 Ub ligases whereas its removal is controlled by deubiquitinating proteases (DUBs), the vast majority of which are cysteine proteases.

The ubiquitin-proteasome pathway has provided a successful therapeutic target in cancer biology. Proteasome inhibitors preferentially induce apoptosis in actively replicating cells and appear particularly effective in the treatment of multiple myeloma (Voorhees and Orlowski, 2006). There is increasing evidence that certain non-eukaryotic pathogens, including *Herpesviridae* (Kattenhorn *et al.*, 2005; Schlieker *et al.*, 2005), the severe acute respiratory syndrome (SARS) virus (Barretto *et al.*, 2005; Lindner *et al.*, 2005), adenoviruses (Balakirev *et al.*, 2002) and *Chlamydia trachomatis* (Misaghi *et al.*, 2006), encode proteins capable of interfering with this pathway, presumably for immune evasive purposes.

The possibility that *Plasmodium* parasites have developed ways to interfere with host cellular functions is an interesting one, particularly in light of how long this parasite has had to evolve. *Plasmodium* has a complex system for the export of membrane proteins to the red blood cell (RBC) surface (Marti *et al.*, 2004) and also secretes non-membrane-associated proteins from micronemes, rhoptries and dense granules (three types of specialized secretory organelles) into the RBC, which are essential for merozoite invasion (Binder and Kim, 2004). Whether these parasites secrete proteins capable

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A Vinylmethylester reaction mechanism



B Vinylmethylsulphone reaction mechanism



* Active site cysteine

** Non-cleavable thioether bond

Fig. 1. Probe mechanisms of action. Probes were designed for the detection of enzymes capable of removing Ub and other Ub-like proteins. An example of the Ub-based probes is shown. Probes include a Ub moiety followed by either (A) a vinylmethylester or (B) a vinylmethylsulphone active group. Where needed, a haemagglutinin (HA) epitope tag is also included as an N-terminal fusion. The catalytic groups act as electrophiles mimicking the C-terminal end of the Ub gly motif and attracting DUB attack. DUBs become covalently and irreversibly attached to the probes through a thioether bond, thereby allowing for detection by anti-HA antibodies in addition to a 10 kDa increase in size.

of interfering with host ubiquitination in nucleated cells remains to be determined. As a eukaryote, *Plasmodium* possesses its own post-translational modifying machinery, including a ubiquitin-proteasome pathway. This pathway has yet to receive its due attention for therapeutic development, with the exception of a recent publication that reports toxicity of proteasome inhibitors for *Plasmodium falciparum* (Lindenthal *et al.*, 2005). Although the complete sequencing of the *P. falciparum* genome has revealed several putative Ub C-terminal hydrolases, there has been no evidence of expression and function for any of these gene products. The assignment of a putative deubiquitinating enzyme based solely on sequence similarity is precarious, as the enzyme may prefer to react with Ub-like modifiers, rather than with Ub itself (Hemelaar *et al.*, 2004; Misaghi *et al.*, 2006). Examples of enzymes with dual specificity, for example, reactive with both Ub as well as with Nedd8, have been reported (Wada *et al.*, 1998; Linghu *et al.*, 2002). Here we identify a *P. falciparum* protein, homologous to human UCH37, which we designate PfUCH54, with apparent deubiquitinating as well as deNeddylating activity.

Results

Use of electrophilic probes to identify modified proteins

Electrophilic derivatives of Ub, SUMO, Nedd8 and ISG15 are activity-based probes that allow identification of enzymes capable of removing these modifications from target proteins (Borodovsky *et al.*, 2001; 2002; Hemelaar *et al.*, 2004). These probes were equipped with epitope

tags to facilitate detection, and their validation and use have been described in detail (Borodovsky *et al.*, 2002; Hemelaar *et al.*, 2004; Kattenhorn *et al.*, 2005; Misaghi *et al.*, 2006). Briefly, these probes comprise the post-translational modifier of choice (Ub, SUMO1, Nedd8 or ISG15) with the C-terminal glycine replaced by an electrophilic vinylmethylester (VME) or vinylmethylsulphone group. Upon attack by the target enzyme, an irreversible thioether bond is formed between enzyme and probe. These interactions can then be detected by immunoprecipitation or by immunoblotting against the epitope tag (Fig. 1).

A search of the *P. falciparum* database predicted a significant number of putative C-terminal Ub hydrolases (Table 1) inciting further investigation into existence and

Table 1. Putative deubiquitinating enzymes in the *Plasmodium falciparum* genome.

Gene ID	Gene name
NP_703247	Ubiquitin C-terminal hydrolase, putative
NP_702688	Ubiquitin-specific protease, putative
NP_702789	Ubiquitin C-terminal hydrolase a, putative
NP_703511	Ubiquitin C-terminal hydrolase 2, putative
NP_703615	Ubiquitin C-terminal hydrolase, putative
NP_704193	Ubiquitin C-terminal hydrolase, putative
NP_701037	Ubiquitin C-terminal hydrolase, family 1, putative
NP_705079	Ubiquitin C-terminal Hydrolase-like zinc finger protein
NP_702465	Ubiquitin C-terminal hydrolase, putative
NP_704194	Hypothetical protein
NP_704848	Hypothetical protein
NP_704154	Hypothetical protein
NP_704195	Hypothetical protein
NP_704588	Hypothetical protein

A *P. falciparum* genome database search yields a short list of putative gene products with predicted deubiquitinating activity.

reactivity profiles for this family of enzymes in the parasite. The probes used in this study are all based on the mammalian sequences of Ub, Nedd8, SUMO1 and ISG15. Consequently, such probes are expected to react only if the *Plasmodium*-derived modifiers are sufficiently conserved to allow cross-reaction. A sequence alignment and consensus analysis of *P. falciparum* SUMO, Nedd8 and Ub modifiers with their human counterparts is shown in Fig. S1. ISG15 homologues have not been found outside of vertebrates so this alignment is not included. A SUMO-related sequence is found in *P. falciparum* and shares an average of 50% amino acid sequence identity with the four human SUMO variants (48% SUMO1, 53% SUMO2, 51% SUMO3, 50% SUMO4). Likewise, a Nedd8-like protein which is 52% identical to its human counterpart is also present. *P. falciparum* and human Ub, however, differ only by a single conservative amino acid replacement (E16D).

Evidence of Ub and Nedd8 protease activity in *P. falciparum*

To determine whether *P. falciparum* contains proteins that have deubiquitinating, deNeddylating, deISG15ylating and deSUMOylating activity, we prepared schizont lysate from synchronized cultures. We chose schizonts, as this stage occupies the entire volume of the RBC and contains large amounts of protein. We treated the cultures with saponin to release the schizonts and to remove any unparasitized red blood cells (uRBCs). Parasites were then lysed and incubated with each of the epitope-tagged probes. Immunoblotting revealed polypeptides reacting with Ub and Nedd8 (Fig. 2A and B). In both cases, addition of *N*-ethyl-maleimide (NEM) eliminated this reactivity, evidence of an enzymatic mechanism that involves a cysteine residue at the active site. Conversely, the SUMO and ISG15 probes failed to react with the parasite lysate (data not shown). The 50% sequence identity of *P. falciparum* SUMO with its mammalian counterpart could account for the absence of reactivity with the mouse-derived probe.

To ensure that the reactive bands were not RBC-derived, we included uRBC controls wherein we reacted uRBC lysate with the Ub and Nedd8 probes. Blotting with anti-haemagglutinin (HA) or anti-FLAG antibodies confirmed that the majority of the strongly reactive material was indeed of parasitic origin.

Identification of Ub-VME-reactive proteins

In order to further characterize these reactive polypeptides, we performed the reaction with HA-Ub-VME on a preparative scale. Parasite lysate was reacted with probe, followed by an anti-HA immunoprecipitation and SDS-

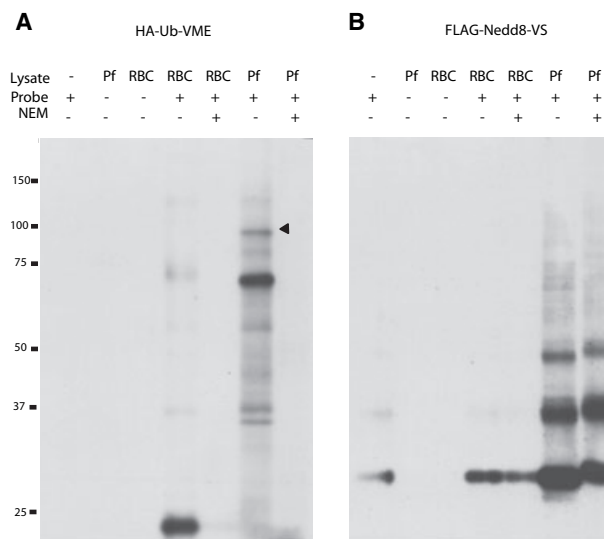


Fig. 2. Evidence of parasite-specific deubiquitinating and deNeddylating activity. Parasite and uninfected red blood cell (RBC) lysates were reacted with (A) HA-Ub-VME or (B) FLAG-Nedd8-VS followed by SDS-PAGE and immunoblot with anti-HA-HRP or anti-FLAG-HRP where appropriate. Probe alone and lysate alone are shown in the left three lanes of each panel. Reaction profiles in the presence or absence of NEM are shown in the right four lanes of each panel. Numbers on the left-hand side of (A) indicate molecular weights in kDa. The reactive polypeptide later excised for mass spectrometric analysis is indicated by the arrow. Pf, *Plasmodium falciparum* lysate.

PAGE. In this instance we opted for unsaponized parasites as the starting material in order to maximize the chance of detecting both internal as well as possibly secreted material that might react with the probe. As a negative control, we included an equal number of uRBCs treated in parallel. The preparative gel was silver-stained, and polypeptides specific to the schizont lysate were excised, trypsinized and subjected to tandem mass spectrometry (MS/MS) (Fig. S2). The corresponding area in the uRBC lane was also excised and analysed to ensure parasite specificity.

Of particular interest was a polypeptide running at approximately 100 kDa, the peptides of which were absent from the uRBC control lane and which matched a protein (Pf11_0177 or NP_701037) annotated as a putative C-terminal Ub hydrolase. The quality of the mass spectra obtained and the extent of sequence coverage allowed robust and unambiguous identification (Fig. 3). Although the gene designation suggests DUB activity, as determined by sequence similarity with known mammalian UCH-type enzymes, these data are the first evidence that this gene product is both expressed and functional. For this reason, the identified protein was named PfUCH54, for *P. falciparum* Ub C-terminal hydrolase with a molecular mass of 54 kDa. The discrepancy in predicted versus observed size of the protein (54 kDa versus

1 MARDNENILE EWCLIESNPC IFYDMLKRMG ATEISVEDVY SLSYFDDYIN
 51 NKEIINMNIH LGVDTYLGEN NK TLDKENNV VDVIELYKNN ICMEDKYNKL
 101 LKHHSYIYGI IFLFNIGKHY KNNK YIEHNV PDNLFFAKQV IPNACATQAI
 151 LSIVLNKDIE LNDEIKNIKT FSLN FDSSMK GLTLSNCTFL RNIHNSYKPP
 201 IYLDKEDVHH DKKKSSEDSFH FVSYISFQDK VYLLDGLQSG PVLINADEQN
 251 KPNPNNNNNN KDNDNDNNNN NNNNNNNNNN NNNNNNNNNN NNIGMNGKDW
 301 IEISREHIKK EIDEICNSQT NNDVRFNIIA VMKDKEYIIQ EYINIHRIVK
 351 QRVNIKLINL GENIELSDEI NEDEFLLND IPSIENLPNN VDTLYNIVNK
 401 STLEINYLQS LLHEQKEIKK LWNKELTFKF FNFYPFIMSS LNLMAKHLL
 451 KDAYQKEKLL NATKS

Coverage:

Sequence	MH+	% Mass	AA	%AA
TLDKENNV VDVIELYK	1892.00	3.48	73-88	3.44
YIEHNV PDNLFFAK	1706.85	3.14	125-138	3.01
FDSSMKGLTL	1098.55	2.02	175-184	2.15
EIDEICNSQT NNDVR	1749.77	3.21	311-325	3.23
Totals:	5310.59	9.76	45	9.68

~100 kDa) can possibly be explained by aggregation in the course of electrophoresis. It has been established that consecutive repetitions of a single amino acid, such as glutamine or alanine, can lead to protein aggregations (Ravikumar *et al.*, 2002). We believe that the homopolymeric region of asparagines embedded in the PfUCH54 sequence may cause the observed aggregation. Peptides matching PfUCH54 were also recovered from 65 kDa and 40 kDa regions of the gel. The 65 kDa mass is consistent with a 54 kDa protein with a 10 kDa probe attachment, while the 40 kDa fragment most likely represents a degradation product. Mass spectral quality for these polypeptides was inferior to that of the 100 kDa band, hence, the latter data are presented in Fig. 3.

When a BLAST database homology search was performed (use of BLOSUM62 matrix, gap costs for existence 11 and for extension 1; low-complexity filter), the human proteasome-associated DUB, UCH37, was identified as a homologue (*e*-value of 1×10^{-30}) despite its significantly smaller size. A sequence alignment of these two proteins, in addition to two other well-characterized human DUBs, UCH-L1 and UCH-L3, revealed only moderate sequence identity but conservation of all active site residues, consistent with the observed DUB activity of the parasitic protein (Fig. 4A). The predicted active residues for PfUCH54 are Cys145, His220 and Asp235. The insertion of a homopolymeric asparagine stretch in the parasite sequence is common to many expressed proteins (Pizzi and Frontali, 2001) in *P. falciparum*. Although the function of these low-complexity homopolymeric regions is not known, they may serve as sites of interaction with other proteins or may expand the parasite's antigenic repertoire, thereby contributing to immune evasion (Schofield, 1991). As the crystal structure of UCH-L3 has been solved (Johnston *et al.*,

1997; Misaghi *et al.*, 2005) and as the structure of UCH37, the closest PfUCH54 homologue, is predicted to be very similar (Kelley *et al.*, 2000), we prepared a threaded model of PfUCH54 based on that of UCH-L3 (Fig. 4B). The predicted structure of the PfUCH54 insertions were calculated using ROSETTA (Bystruff and Shao, 2002) and manually inserted into the final model (Guex and Peitsch, 1997). We were encouraged to see that the three major insertions in the parasitic protein sequence (shown in green in the model) are unlikely to interfere with the predicted architecture of the catalytic site (active cysteine residue shown in blue), as was confirmed by experiment (see Fig. 5). If these homopolymeric amino acid stretches serve a function, it is likely one not directly related to the protein's enzymatic activity.

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Parasitic DUB also has deNeddylating activity

To verify DUB activity for this protein and to probe the extent of possible deNeddylating, deSUMOylating and delSG15ylating activity, the PfUCH54 coding region (which does not contain introns) was cloned from genomic DNA and a catalytically inactive mutant was generated based on this sequence by mutating the putative active site cysteine residue to serine. Both constructs were epitope-tagged with HA and their expression and reactivity with the probes were assessed by *in vitro* translation.

Although the *in vitro* translation product failed to react with the SUMO and ISG15 probes, modification by Ub-VME was evident from a quantitative shift of the protein to a more slowly migrating form, consistent with covalent adduct formation with the probe (Fig. 5). Furthermore, a similar shift was also observed with the Nedd8-vinylsulphone (VS) probe. Based on reactivity with these

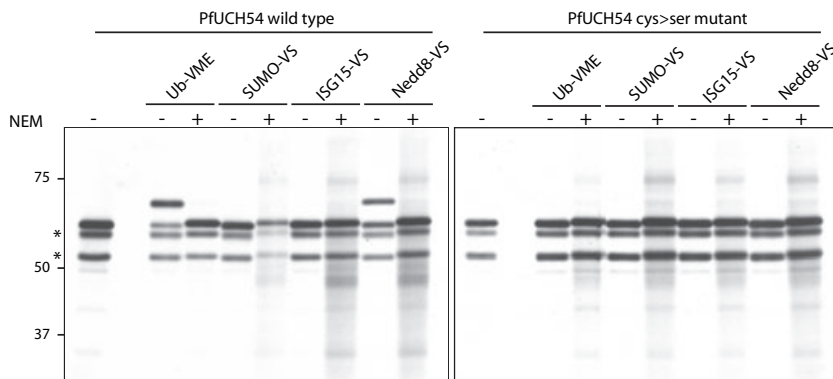


Fig. 5. Evidence of dual Ub and Nedd8 specificity for PfUCH54. *In vitro* transcription/translation using HA-tagged, wild-type PfUCH54 (left) and the catalytically inactive cysteine to serine mutant (right) as templates was performed in the presence of ^{35}S -methionine. Translation products were reacted with the range of untagged probes followed by immunoprecipitation against HA. Analysis by autoradiography reveals Ub and Nedd8 activity specific to the wild-type PfUCH54. Two unreactive truncated translation products are indicated by asterisk. Reactivity in the presence or absence of the alkylating agent NEM is shown for both the wild type and the mutant.

probes, enzymatic activity is inferred (Kattenhorn *et al.*, 2005; Schlieker *et al.*, 2005; Misaghi *et al.*, 2006). Binding to both Ub-VME and Nedd8-V5 were entirely blocked upon addition of NEM. Specificity was further substantiated by the failure of the catalytically inactive mutated protein to be modified by either probe. These data demonstrate the requirements for the cysteine residue for reactivity, a hallmark of DUB activity. In addition to the polypeptide that exhibits mobility shifts upon reaction with the probe, each sample contains two additional prominent polypeptides that do not react with the probe. Because these polypeptides are recovered via the C-terminal epitope tag, they must correspond to N-terminal truncations of the primary translation product. Based on the location of additional methionine residues at positions consistent with the observed size of these polypeptides, we propose that they derive from the use of alternative translation start sites (indicated in Fig. 3).

Discussion

The identification of molecules and metabolic pathways unique to *P. falciparum*, or sufficiently distinct from its human host to allow chemical discrimination, may yield new targets for the development of antiparasitic vaccines and therapeutic agents. Differences in the ubiquitin-proteasome system have been successfully used for diagnosis of parasitic diseases (Telles *et al.*, 2003). The ubiquitin-proteasome pathway is ancient and has been conserved throughout evolution (Catic and Ploegh, 2005), and the dynamic addition and removal of Ub is central to a wide variety of eukaryotic cellular processes in organisms ranging from yeast to humans. The many effects of ubiquitination render the enzymes that act on Ub crucial to proper cellular function and survival, a possible target for development of methods to handicap pathogens. The evolutionary distance between the parasite and its host may have resulted in differences in enzyme structure sufficient to allow selective inhibition, with the core function of the enzyme remaining conserved. Interference with

the ubiquitin-proteasome pathway has been successfully targeted in cancer therapy (Voorhees and Orłowski, 2006) but has been largely overlooked within infectious disease, particularly with regard to diseases of significant public health importance, such as malaria. Indeed, interference with protein degradation in *P. falciparum*, by blocking proteasome function, results in growth inhibition and toxicity to the parasite (Gantt *et al.* 1998; Lindenthal *et al.*, 2005) and validates this pathway as a legitimate drug target. Given the variety of molecules that mediate this branch of the ubiquitination pathway, more specific and elegant therapies might be developed by targeting individual components of the processing cascade, rather than by indiscriminately blocking the proteasome itself. The data presented here establish the expression and functionality of the enzyme PfUCH54, both as a deubiquitinating and as a deNeddylating agent and provide the first experimental evidence that these enzyme families are active in *P. falciparum*.

The dual deubiquitinating and deNeddylating activity of PfUCH54 is reminiscent of UCH-L3 dual reactivity (Wada *et al.*, 1998) and has been reported for other enzymes as well (Linghu *et al.*, 2002; Misaghi *et al.*, 2005), suggesting that this dual activity may be a widespread, conserved and significant phenomenon. Although the alignment of UCH-L3 with PfUCH54 does not show a high level of identity between the two proteins, putative active site amino acids reside within highly conserved sequence blocks. The dual specificity observed for PfUCH54 does not allow us to state with confidence whether its primary function is to target Ub-modified or Nedd8-modified proteins. Nedd8's role, as far as is known, is restricted to the modification of cullins, which are part of the SCF-type Ub ligases involved in cell cycle control (Chiba and Tanaka, 2004). It is possible that PfUCH54 may contribute to regulation of both Ub and Nedd8 conjugation in *P. falciparum*. The Nedd8 reactivity of PfUCH54 is of particular interest given that mammalian and parasitic Nedd8 share only 52% identity. Nonetheless, there are three distinct blocks of conserved sequence (aa 5–11, 42–50,

71–76; see Fig. S1), which suggests that these regions of the protein are the critical components for proper folding and function.

Non-eukaryotic pathogens such as viruses and bacteria in some cases express proteins that can catalyse deubiquitination, presumably as part of immune evasion strategies. These proteins are either assembled or secreted into the host cell's cytoplasm where they interfere with the Ub pathway. A catalytically active truncation of UL36, the major tegument protein of herpes simplex virus-1 (HSV-1), is able to cleave polyubiquitin chains (Kattenhorn *et al.*, 2005) and a related activity is conserved across the *Herpesviridae* (Schlieker *et al.*, 2005). Likewise, *C. trachomatis* encodes two recently identified proteins with deubiquitinating activity, ChlaDUB1 and ChlaDUB2 (Misaghi *et al.*, 2006). Homologues to these enzymes are not present in non-pathogenic *Chlamydia* spp., an observation that supports the idea that these proteins may contribute to pathogenicity. Additional examples of proteins with deubiquitinating activity are also encoded by the SARS virus (Barretto *et al.*, 2005; Lindner *et al.*, 2005) as well as adenoviruses (Balakirev *et al.*, 2002).

As a major aspect of *P. falciparum* pathogenesis is mediated by the parasite's ability to display its own proteins on the surfaces of host erythrocytes, the mechanisms surrounding secretion have been well studied (Sargeant *et al.*, 2006). In order for a parasite protein to be secreted, it must contain a specific N-terminal signal sequence, otherwise known as a PEXEL domain (Binder and Kim, 2004; Marti *et al.*, 2004). PfUCH54 lacks such a sequence, suggesting that its function is intraparasitic rather than host-targeted. In addition, asparagine repeats which are commonly found throughout the *P. falciparum* genome are most commonly associated with parasite-internal proteins (Singh *et al.*, 2004). The presence of an asparagine-rich region in PfUCH54 and the presence of a strongly HA-Ub-VME-reactive band at 54 kDa in saponized parasites (Fig. 2A) suggest intraparasitic localization, a conclusion that would require further support by direct immunohistochemical analysis.

The significance and necessity of PfUCH54 and other Ub-reactive proteins in *P. falciparum* remain to be determined. Efforts to assess parasite viability in the absence of these molecules are underway in the form of knockout and mutation studies in live parasites. Unlike in other organism, there seem to be few transcriptional changes in *P. falciparum* following exposure to stimuli such as antifolates (Ganesan *et al.*, 2003). This rigidity in transcription suggests that post-transcriptional regulation, including protein turnover, is likely to play a major role in parasite gene expression and parasite viability. By targeting specific DUBs, we hope to open new avenues in the development of antimalarial therapeutics.

Experimental procedures

Parasite culturing and purification

Plasmodium falciparum parasites (3D7 strain) were maintained in culture as previously described (Trager and Jensen, 1976). Mature schizonts were isolated from synchronous cultures with a parasitaemia of 4% by two methods. For one sample, the infected RBC pellet was simply washed twice with PBS before storage at -80°C . In the other sample, the infected RBC pellet was lysed in an incubation of 0.15% saponin to release the mature schizonts before subsequent washing in PBS and storage at -80°C .

Cloning and mutagenesis

Plasmodium falciparum genomic DNA was prepared using the Blood and Body Fluid Spin Protocol in Qiagen's QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook according to manufacturer's specifications (Valencia, CA). Starting parasite material was prepared by incubating 8×10^7 parasitized RBCs of mixed stages with saponin (0.15%) for 10 min at room temperature. Released parasites were centrifuge-pelleted at 500 *g* for 5 min and washed in chilled PBS twice. Subsequently, supernatant was removed and pellets were flash frozen and stored at -80°C .

Pf11_0177 is the intronless, open reading frame on chromosome 11 that encodes PfUCH54. Primers were designed against the 5' (CGGAATTCATGGCGAGGGATAATGAAACA) and 3' (CCGCTCGAGAGATTTTGTTCATTTTTTAATTT) regions of the open reading frame with EcoRI and XhoI linkers, respectively, for directional cloning. The sequence was PCR-amplified with Pfu polymerase and the product was digested and ligated into the multicloning site of pcDNA3.1(+) in frame with a C-terminal HA epitope tag. The ligation mixture was transformed to competent *Escherichia coli* and colonies harbouring a plasmid were picked for DNA isolation. Clones positive for an insert of the correct size were selected and their DNA sequence was verified. Correct clones were used for subsequent studies.

Site-directed mutagenesis was performed using Stratagene's QuikChange II Site-Directed mutagenesis Kit (La Jolla, CA). Complementary primers spanning the area immediately around the catalytic cysteine codon were designed (5' > 3' GTTATACCAATGCAAGTGCTACACAAGC and 3' > 5' GCTTGTGTAGCACTTGCATTTGGTATAAC) to change the cysteine to a serine by mutation of a single base (indicated by the underlined nucleotides). Thirty cycles of PCR followed by DpnI digestion to remove the methylated template plasmid DNA yielded product which was transformed to competent *E. coli* as described above. Properly mutated clones were identified by sequence verification.

Probes

Haemagglutinin- or FLAG-tagged, VME or VS Ub, Nedd8, ISG15 and SUMO electrophilic probes were previously generated and described (Borodovsky *et al.*, 2001; 2002; Hemelaar *et al.*, 2004). Reactions with parasite lysate were carried out for 90 min at a concentration of approximately 0.3 μg of probe for every 1 μg of parasite protein. For the

immunoblot the lysed equivalent of 5×10^7 schizonts or uRBCs were loaded per lane. For the preparative gel, 4 mg of total lysed protein (derived from 12×10^{10} RBC containing 2.5×10^9 schizonts or 12×10^{10} uRBC) was reacted with 40 μ g of probe.

Immunoblot

Parasite lysate was separated by gel electrophoresis and proteins were transferred to PVDF which were blocked overnight in 5% milk. Membranes were then probed with anti-HA-HRP (Roche, Mannheim, Germany) or anti-FLAG-HRP (Sigma, St Louis, MO) antibodies at a dilution of 1:1000 in PBS-Tween for 1 h at room temperature. Following six 10 min washes in PBS-Tween, proteins were visualized by chemiluminescence (Perkin Elmer, Boston, MA).

In vitro translation and immunoprecipitation

Template plasmid DNA of either the wild-type or mutated gene was added at a concentration of 200–300 μ g ml⁻¹ to 30 μ l of rabbit reticulocyte lysate TNT T7 Quick Coupled Translation/Transcription System mastermix (Promega, Madison, WI). Each reaction was supplemented with 1 μ Ci of ³⁵S-methionine (Perkin Elmer) and incubated at 30°C for 1 h. Each sample mixture was then reacted with 0.3 μ g of untagged probe (either Ub-VME, Nedd8-VS, ISG15-VS or SUMO-VS) for 90 min at room temperature and then diluted to a 1 ml volume in NP-40 lysis buffer [10 mM Tris (pH 7.8), 150 mM NaCl, 5 mM MgCl₂, 0.5% NP-40]. Enzyme–probe complexes were immunoprecipitated by 12CA5 mouse monoclonal anti-HA antibody for 45 min at 4°C followed by addition of Immobilized Protein-A agarose (Repligen, Waltham, MA) for another 45 min at 4°C. Beads were spun and washed in washing buffer [0.5% NP-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA] three times, resuspended in reducing sample buffer and the eluate was subjected to SDS-PAGE (8% polyacrylamide). Results were analysed by autoradiography.

Modelling

A homology model of PfUCH54 was constructed based on the solved structure of UCH-L3, and predicted secondary structure of the *Plasmodium* protein. Inspection of sequence alignment of PfUCH54 with UCH-L1, UCH-L3 and UCH37 shows regions of similarity in all four proteins that are punctuated by large (> 25 amino acids) insertions in PfUCH54. These insertions were omitted from the initial modelling as they are present in regions of UCH-L3 that are not part of the Ub binding site or the active site of UCH-L3.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Alignment of human and *P. falciparum* Ub and Ub-like proteins. The extent of amino acid sequence homology between human and *P. falciparum* (A) SUMO variants (B) Nedd8 and (C) Ub. Solid blocks indicating regions of conserved amino acids. Amino acid sequences were obtained from the GenBank database. The accession number are as follows: SUMO GI:23504543, Nedd8 GI:23615283, Ub GI:23508814.

Fig. S2. Preparative silver-stained gel for mass spectrometric analysis. Lysates derived from 12×10^{10} RBC containing 2.5×10^9 schizonts or 12×10^{10} uRBC were reacted with HA-Ub-VME probe. Reactive material was immunoprecipitated with anti-HA resin, SDS-PAGE separated, and polypeptides indicated by the arrows were excised and subjected to tandem mass spectrometry. PfUCH54-matching peptides were found in bands indicated by the asterisks.

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