Identification by functional proteomics of a deubiquitinating/deNeddylation 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 deNeddylation 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
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 electrophile mimicking the C-terminal end of the Ub gly motif and attracting DUB attack. 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 activity.

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, delSG15ylating 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-PAGE. In this instance we opted for unsaponized parasitess 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...
The structure of UCH-L3 has been solved (Johnston et al., 1997; Misaghi et al., 2005) and as the structure of UCH37, the closest PIUCH54 homologue, is predicted to be very similar (Kelley et al., 2000), we prepared a threaded model of PIUCH54 based on that of UCH-L3 (Fig. 4B). The predicted structure of the PIUCH54 insertions were calculated using ROSETTA (Bystroff 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.

**Parasitic DUB also has deNeddylating activity**

To verify DUB activity for this protein and to probe the extent of possible deNeddylating, deSUMOylating and deISG15ylating activity, the PIUCH54 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 translation product. PIUCH54 by mass spectrometry. *P. falciparum* and uninfected erythrocyte lysates were reacted with HA-Ub-VME probe on a preparative scale followed by SDS-PAGE. Parasite-specific reactive proteins were extracted and subjected to tandem mass spectrometry. Identified polypeptides (indicated in pink) were screened against the P. falciparum genome database and matched a 465-amino-acid protein annotated as a putative C-terminal Ub hydrolase. Mass spectrometry properties of the identified peptides are shown. MH+ indicates the protonated mass of each peptide; %Mass represents the per cent of the total protein mass occupied by each peptide; AA identifies the span of amino acids covered by each peptide; and %AA identifies the per cent of amino acid coverage. Black arrows indicate potential alternative internal start sites as determined by additional translation products seen in Fig. 5.
Fig. 4. Alignment of PfUCH54 with human DUB homologues and predicted structure.
A. An alignment of three human UCH-type DUBs, UCH-L1, UCH-L3 and UCH37, with PfUCH54 is shown with active residues indicated by the blue arrows.
B. The crystal structure of UCH-L3 was used to model PfUCH54. Parasite-specific insertions are shown in green. Active site cysteine residues for both enzymes are shown in blue. The arrow indicates the trajectory of Ub that is presumed for binding to UCH-L3.

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probes, enzymatic activity is inferred (Kattenhorn et al., 2005; Schlieker et al., 2005; Misaghi et al., 2006). Binding to both Ub-VME and Nedd8-VS 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 Orlowski, 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 polyubiquitins (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 PIUCH54 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 PIUCH54 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 × 10⁷ 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 PIUCH54. Primers were designed against the 5′ (CCGAAATTCATGGCGAGGGATAATGAA AACA) and 3′ (CCGGGCAGGATTTGTGCATTTTT TAATT) 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′ GTTATACCAATGACATGGCTACACAGG and 3′ > 5′ GCTTGTGTAAGCCATATTGCATTGTTGAT) 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 electrophoretic 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
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 × 10^8 schizonts or 12 × 10^10 uRBC) was reacted with 40 μg 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 μg ml⁻¹ to 30 μl of rabbit reticulocyte lysate TNT T7 Quick Coupled Translation/Transcription System mastermix (Promega, Madison, WI). Each reaction was supplemented with 1 μCi of 35S-methionine (Perkin Elmer) and incubated at 30°C for 1 h. Each sample mixture was then reacted with 0.3 μg of untagged probe (either Ub-VME, Nedd8-VS, ISG15-VS or untagged probe (either Ub-VME, Nedd8-VS, ISG15-VS or SUMO-VS) for 90 min at room temperature and then diluted with PBS-Tween for 1 h at room temperature. Following six 10 min washes in PBS-Tween, proteins were visualized 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 ¥ 10¹⁰ RBC containing 2.5 ¥ 10⁹ schizonts or 12 ¥ 10¹⁰ 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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