**Plasmodium falciparum** cysteine protease falcipain-1 is not essential in erythrocytic stage malaria parasites


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Among potential new targets for antimalarial chemotherapy are *Plasmodium falciparum* cysteine proteases, known as falcipains. Falcipain-2 and falcipain-3 are food vacuole hemoglobinases that may have additional functions. The function of falcipain-1 remains uncertain. To better characterize the role of falcipain-1 in erythrocytic parasites, we disrupted the falcipain-1 gene and characterized recombinant parasites. Disruption of the falcipain-1 gene was confirmed with Southern blots, and loss of expression of falcipain-1 was confirmed with immunoblots and by loss of labeling with a specific protease inhibitor. Compared with wild-type parasites, falcipain-1 knockout parasites developed normally, with the same morphology, multiplication rate, and invasion efficiency, and without significant differences in sensitivity to cysteine protease inhibitors. In wild-type and knockout parasites, cysteine protease inhibitors blocked hemoglobin hydrolysis in trophozoites, with a subsequent block in rupture of erythrocytes by mature schizonts, but they did not inhibit erythrocyte invasion by merozoites. Our results indicate that although falcipain-1 is expressed by erythrocytic parasites, it is not essential for normal development during this stage or for erythrocyte invasion.

Malaria is one of the most important infectious disease problems of humans, particularly in developing countries. *Plasmodium falciparum*, the most virulent human malaria parasite, is responsible for hundreds of millions of illnesses and more than 1 million deaths each year (1). The control of malaria is increasingly limited by the resistance of malaria parasites to available drugs. New antimalarial drugs, ideally directed against new targets, are needed.

Among potential new targets for antimalarial chemotherapy are cysteine proteases. The best-characterized plasmoidal cysteine proteases are falcipains, which are papain-family cysteine proteases that are expressed by erythrocytic parasites (2, 3) and share many unusual features (3). Two nearly identical copies of falcipain-2 and falcipain-3 are papain-family cysteine proteases that hydrolyze hemoglobin in trophozoites, with a subsequent block in rupture of erythrocytes by mature schizonts, but they did not inhibit erythrocyte invasion by merozoites. Our results indicate that although falcipain-1 is expressed by erythrocytic parasites, it is not essential for normal development during this stage or for erythrocyte invasion.

**Materials and Methods**

**Materials.** *P. falciparum* strains D10, 3D7, HB3, and W2 were obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). The transfection plasmid pDC and compound WR99210 were gifts from David Fidock (9) and David Jacobus (Jacobus Pharmaceuticals, Princeton), respectively. Compound YA29 was a gift from Matthew Boggo (Stanford University, Stanford, CA) and was synthesized as reported in ref. 8. All other biochemical reagents were from Sigma or Fisher.

**Parasite Culture.** *P. falciparum* parasites were cultured in human erythrocytes at 2% hematocrit in RPMI medium 1640 supplemented with 10% human serum (10). Synchronization was maintained by serial treatment with 5% d-sorbitol (11).

**Construction of Transfection Plasmids and Generation of Falcipain-1 Knockout Parasites.** DNA encoding truncated falcipain-1 (ΔFP1; lacking coding sequence for the first 179 N-terminal and the last 37 C-terminal amino acid residues, including a catalytic Asn) was PCR-amplified from the pTOF-FP1 plasmid by using Taq DNA polymerase (Innogenent) and appropriate primers (forward: 5′-AATGCATGCAAGCTTCGTGAAGAAGAAAAGATGAAGAC-3′; reverse: 5′-CCAGTCGACAGCTTATCAATATAGTATATAATATTATCCATCTCGG-3′; *HindIII* sites are underlined). Plasmid pDC contains pCDT5′/HDHFR/Phfrp2-3′ and cam5′/hsp86-3′ expression cassettes for selection with WR99210 and expression of the desired gene in *P. falciparum* parasites, respectively (9). The cam5′/hsp86-3′ expression cassette was replaced with *HindIII*-digested ΔFP1 to construct the transfection plasmid pD-FP1. Ring stage D10-strain parasites were transfected with 100 μg of pDC-FP1 (prepared with the Mobius 1000 Plasmid kit from Novagen) and selected with WR99210 as described in refs. 6 and 12. Genomic DNA (gDNA) was extracted from parasites and analyzed for chromosomal integration of the plasmid by PCR with integration (1UP5′-AAATCACTCGGCCGGCCTCGAGA-TAATAAATAG-3′; MR: M13 reverse primer, Invitrogen) and WT (1UP; 5′-TGTTAAGCCTTTACAAGATAGGATAGAAGC-3′) specific primers. To eliminate
parasites containing episomal plasmids, we subjected cultures to two 2-week cycles of incubation with and without WR99210 (5 nM). We then cloned recombinant parasites by limiting dilution and analyzed multiple clones by PCR for falcipain-1 gene disruption.

**Southern Hybridization.** gDNA was isolated from schizont-stage parasites by using the PureGene DNA isolation kit (Gentra Systems), digested with XmnI or HincII–BsrGI restriction endonucleases, separated on agarose gels, transferred to Hybond-N membranes (Amersham Pharmacia Biosciences), and probed with [α-32P]dATP-labeled truncated falcipain-1 (Mega-prime DNA labeling system, Amersham Pharmacia Biosciences), as described in ref. 5.

**Immunoblotting.** Parasites were harvested at 6–10% parasitemia at 12-h intervals; parasite pellets were suspended in 200 μl of PBS [with 1 mM PMSF, 5 mM EDTA, 2 mM benzamidine–HCl, 10 μM pepstatin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)], and protein extracts were resolved by SDS/PAGE (13) and transferred onto poly(vinylidene difluoride) membranes (Bio-Rad). Membranes were incubated with blocking buffer (PBS/0.2% casein/0.1% Tween 20) containing appropriate dilutions of antibodies to purified recombinant falcipains [mature falcipain-1, truncated-profalcipain-2, and truncated-profalcipain-3 (5)], followed by alkaline phosphatase-conjugated goat anti-rat IgG in blocking buffer (1/5,000 dilution), and reactions were developed by using a Western-Star chemiluminescence kit (Tropix, Bedford, MA).

**Measurement of Parasite Development.** Synchronized parasites were cultured in complete medium for three cycles with change of medium after every cycle. Every 48 h, when parasites were at the ring stage, triplicate aliquots were fixed with 1% formaldehyde in PBS (pH 7.4) for 48 h at room temperature and labeled with YOYO-1 (1 nM; Molecular Probes) in 0.1% Triton X-100 for flow cytometry, as described.

**Test of Proplasmepsin Processing.** Trophozoite-stage PM2GT B7 parasites expressing a plasmepsin II– GFP fusion (15) were transfected with a pD-FP1 plasmid carrying a truncated falcipain-1 gene and a PcDT5′/hDHFR/Pfhrp2-3′ expression cassette for selection with WR99210 (Fig. 1). After 12 weeks of culture with WR99210, plasmid integration at the falcipain-1 locus was verified by PCR. Parasites then were subjected to two rounds of selection with and without drug, followed by cloning by limiting dilution to obtain a homogenous population. Two falcipain-1 knockout clones (KO2 and KO11) were studied in detail. PCR with WT and integration-specific primers indicated integration of the plasmid into the FP1 locus and absence of the intact falcipain-1 gene (Fig. 24). Southern blotting showed genomic restriction digestion patterns for each clone consistent with disruption of the falcipain-1 locus by single-site homologous crossover-mediated insertion of three copies of the plasmid (Fig. 2B). Immunoblotting with monospecific antibodies to falcipains confirmed the complete absence of falcipain-1 expression in recombinant parasites, whereas expression of falcipain-2 and falcipain-3 remained very similar to that in WT parasites (Fig. 2C) (5). As final confirmation of the knockout, binding of the generic cysteine protease inhibitor 125I-labeled DCG04 to a protein previously identified as falcipain-1 (8) was seen in the parental strain, but not recombinant parasites (M. Bogyo, personal communication).

**Characterization of Falcipain-1 Knockout Parasites.** The morphology of WT and falcipain-1 knockout parasites was the same throughout the life cycle. The knockout parasites showed growth patterns very similar to those of WT parasites; after three cycles of growth parasitemias were nearly identical for the WT strain and two knockout clones (Fig. 3A). Knockout and WT parasites also exhibited nearly identical rates of invasion of erythrocytes (Fig. 3B). For all three lines, ring parasitemias increased 4- to 5-fold over the schizont parasitemias of the prior cycle.

**Effects of Cysteine Protease Inhibitors on Erythrocyte Rupture and Invasion.** Broadly active cysteine protease inhibitors such as E-64 are known to block parasite development by inhibiting hemo-
globin hydrolysis (18, 19) and possibly by action against erythrocyte rupture or invasion (18, 20–22). Recently, a competition analysis evaluating effects of a peptidyl epoxide inhibitor library against *P. falciparum* lysates identified a falcipain-1-specific inhibitor, YA29. This compound was reported to inhibit erythrocyte invasion by merozoites, suggesting a specific function for falcipain-1. However, the hypothesis that falcipain-1 mediates erythrocyte invasion was not consistent with prior studies that did not show the inhibition of erythrocyte invasion by cysteine protease inhibitors (20) or our result that falcipain-1 knockout parasites had normal invasion rates. To clarify the effects of YA29, we evaluated the morphologies of 3D7-strain parasites after incubations with YA29 beginning at the late ring (16 h postinvasion) or schizont (40 h postinvasion) stages (Fig. 4). In both cases, parasites developed swollen, dark-staining food vacuoles, consistent with an inhibition of hemoglobin hydrolysis, as described for other cysteine protease inhibitors (17, 18).

We also closely followed the loss of schizonts and formation of rings after incubation of YA29 with early schizonts (Fig. 5A). YA29 caused a modest inhibition of the rupture of schizonts (seen as the accumulation of schizonts, compared with control), beginning at 50 h postinvasion, when schizont rupture and new ring formation were occurring in control parasites. Beginning at 48 h postinvasion, ring parasitemia was lower in treated than control cultures, consistent with the explanation that YA29 exerted a modest inhibition in new ring formation because of its inhibition of normal parasite maturation and possibly independent effects on erythrocyte rupture by mature schizonts. The inhibition of ring formation was more pronounced when YA29 was added earlier in the life cycle (36 or 38 h postinvasion), presumably because earlier incubation exerted more pronounced inhibitory effects on hemoglobin hydrolysis (Fig. 5B).

The peptidyl epoxide YA29 was previously identified as a falcipain-1-specific inhibitor (8), although a direct measure of inhibition was not possible because of the lack of recombinant active falcipain-1. However, recombinant falcipain-2 and falcipain-3 are available. To better appreciate the inhibitory speci-
ficity of YA29, we assessed the actions of it and of other cysteine protease inhibitors against recombinant enzymes and on the development of three *P. falciparum* strains. YA29 showed modest activity against falcipain-2, but not falcipain-3 (Table 1).

Results with E-64 and YA29 were similar for three different strains of *P. falciparum*, including those sensitive (3D7, HB3) and resistant (W2) to chloroquine and other antimalarial drugs (Table 1). Curiously, the more membrane-permeant E-64 analog, E-64d, was 4 times more effective against W2-strain parasites than against drug-sensitive strains.

Another predicted cysteine protease activity of erythrocytic parasites is the processing of plasmepsin aspartic proteases (15, 23, 24). Inhibition of this activity in parasites transfected with GFP-tagged plasmepsin II led to the accumulation of unprocessed fluorescent plasmepsin II in the food vacuole membrane, which appeared as a fluorescent ring surrounding the food vacuole (15). This unique distribution of GFP-tagged plasmepsin II was caused by the peptidyl inhibitor ALLN, but not E-64 (16). To determine whether the effects of YA29 were due, at least in part, to inhibition of plasmepsin II processing activity, we treated parasites expressing GFP-tagged plasmepsin II with ALLN, E-64, and YA29, and evaluated the morphology of live parasites by fluorescence microscopy. As with E-64, YA29 did not cause the morphology that is indicative of an inhibition of plasmepsin processing (Fig. 6). Therefore, it does not appear that the inhibitory effect of YA29 was because of its action on the processing of plasmepsins.

**Sensitivity of Falcipain-1 Knockout Parasites to Protease Inhibitors.**

We next compared the effects of cysteine protease inhibitors and other compounds on the development of WT and knockout parasites. Cysteine protease inhibitors were somewhat more active against falcipain-1 knockout parasites than against WT, although in all cases changes in sensitivity were not statistically significant (Fig. 7). Interestingly, knockout clone KO11 was more sensitive than clone KO2 to E-64 and E64d. The aspartic protease inhibitor pepstatin was equally active against knockout and parental parasites. The antimalarial drug chloroquine was

**Table 1. Inhibition of recombinant protease activity and parasite multiplication**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Falcipain-2</th>
<th>Falcipain-3</th>
<th>3D7</th>
<th>W2</th>
<th>HB3</th>
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<tr>
<td>E-64</td>
<td>0.012</td>
<td>0.032</td>
<td>2.9</td>
<td>2.6</td>
<td>2.1</td>
</tr>
<tr>
<td>E-64d</td>
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<td>6.3</td>
<td>1.9</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>YA29</td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>9.4</td>
<td>8.8</td>
<td>21.1</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>ND</td>
<td>ND</td>
<td>8.3</td>
<td>6.8</td>
<td>6.3</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>ND</td>
<td>ND</td>
<td>0.01 &gt;0.10</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

*All values are mean IC50, based on three replicates for each inhibitor, with all experiments performed with the same concentration of enzyme.

†Mean IC50 values were determined from two experiments, each done in duplicate.
cultures and are expressed as percentage of the number of schizonts at the number of unruptured schizonts in a DMSO control from those in treated means of two replicates. Counts of schizonts were corrected by subtracting the rings (A concentrations of the inhibitors for 12 h. Counts of schizonts (40 h postinvasion) were combined with fresh erythrocytes (2% hematocrit, 2 P. falciparum

Discussion

New transfection technologies allow us to precisely examine the functions of P. falciparum proteins. Knockout strategies are particularly attractive for defining the functions of enzymes, such as falcipain-1, for which heterologous expression systems are not well developed. The knockout of falcipain-1 was readily achieved, in part because the loss of this protein did not confer a significant disadvantage to the parasite. Falcipain-1 knockout parasites had normal morphologies and multiplied at the same rate as WT parasites. Thus, falcipain-1 is not essential for erythrocytic stage parasites. Our results do not exclude a potential role for falcipain-1 in erythrocytic parasites, however, because up-regulation of other proteases may have compensated for loss of falcipain-1 in the knockout parasites.

P. falciparum likely requires protease activity for multiple processes during the erythrocytic cycle, including hemoglobin degradation, the hydrolysis of host and parasite proteins that accompanies erythrocyte rupture and then invasion, and the processing of plasmodial proteins throughout the life cycle. Attempts to dissect the functions of plasmodial proteases have included studies of the effects of protease inhibitors on cultured parasites. Cysteine protease inhibitors block hemoglobin hydrolysis and development by trophozoites, leading to a characteristic morphological abnormality in which the food vacuole fills with undegraded hemoglobin (18, 19). In addition, because of the inhibition of development or independent effects, cysteine protease inhibitors block the release of merozoites from schizont-infected erythrocytes at the completion of the erythrocytic cycle (18, 20–22). In in vitro studies, falcipain-2 and falcipain-3 hydrolyzed hemoglobin (4, 5) and falcipain-2 also hydrolyzed the erythrocyte cytoskeletal proteins ankyrin and band 4.1 (25, 26), but until recently, specific roles of cysteine proteases in the parasite life cycle have been uncertain.

We now appreciate that four typical papain-family cysteine proteases are present in P. falciparum: falcipain-1, two nearly identical copies of falcipain-2, and falcipain-3. Falcipain-2 and falcipain-3 have been well characterized recently, and appear to participate in hemoglobin degradation by trophozoites, as both proteases have been localized to trophozoite food vacuoles, possess acidic pH optima, and degrade hemoglobin in vitro (4, 5). In addition, it was demonstrated recently that disruption of the falcipain-2 gene led to decreased hemoglobin degradation by trophozoites, proving a role for falcipain-2 in this process (6). Attempts to knock out falcipain-3 have to date been unsuccessful (P.S.S., unpublished observation), suggesting that this protease, which unlike falcipain-2 is encoded by a single-copy gene, plays a key role in erythrocytic parasites. Despite falcipain-1 being the first plasmodial cysteine protease gene identified (7), its detailed biochemical characterization has not been possible because of inadequate systems for the production of recombinant protease (27). Recently, a chemical genetics approach was used to circumvent this problem by identifying a falcipain-1 inhibitor (YA29) by using an inhibitor competition assay and then evaluating the biological effects of this inhibitor. This compound appeared to specifically inhibit the invasion of erythrocytes by P. falciparum merozoites, leading to a proposed role for falcipain-1 in this process. However, it remained unclear whether the specificity of YA29 for falcipain-1 was complete. It also is a concern that these results conflicted with prior studies with isolated merozoites that showed no inhibition of erythrocyte invasion by broadly active cysteine protease inhibitors (20, 28).

To further examine the role of falcipain-1 in erythrocytic parasites, we disrupted the falcipain-1 gene. Falcipain-1 knockout parasites developed normally, clearly indicating that this protease is not essential for erythrocytic malaria parasites. The selection of falcipain-1 knockout parasites may have been accompanied by the elaboration of an invasion mechanism different from that in WT parasites. To consider this possibility, the effects of YA29 were studied in detail. Compared with untreated parasites, those treated with YA29 demonstrated an accumulation of unruptured schizonts. Earlier treatment led to a more pronounced inhibitory effect, presumably because of more pronounced inhibition of hemoglobin hydrolysis in trophozoites and early schizonts than in more mature parasites. The concentration of YA29 required to block development and schizont rupture was in the micromolar range, but varied somewhat between experiments, perhaps because of evaluation of different parasite strains and/or minor differences in the stages at which parasites were treated. However, it is important to note that our studies included the same three P. falciparum strains that were previously demonstrated to show a block in erythrocyte invasion after incubation with YA29 (8).

The effects of the broadly active cysteine protease inhibitors E-64 and E-64d did not differ notably from those of YA29, which was identified as a specific falcipain-1 inhibitor but also has modest activity against recombinant falcipain-2. All of the cysteine protease inhibitors blocked hemoglobin hydrolysis in trophozoites and early schizonts and inhibited subsequent erythrocyte rupture by mature schizonts. Our results are consistent
with prior observations that the trophozoite stage is most sensitive to treatment with cysteine protease inhibitors (29) and with the conclusion that the principal activity of these agents is to block hemoglobin hydrolysis. Falcipain-2 and falcipain-3 seem to be the principal hemoglobinases that are acted on by cysteine protease inhibitors, as supported by correlations in many classes of inhibitors between action against these enzymes, inhibition of hemoglobin hydrolysis, and prevention of parasite development (16, 17, 30, 31). It remains unclear whether effects of cysteine protease inhibitors on erythrocyte rupture were due only to the consequences of the inhibition of hemoglobin hydrolysis, or whether inhibition of other activities of cysteine proteases also played a role. Cysteine protease-mediated activation of plasmodin aspartic proteases also may contribute indirectly to hemoglobin hydrolysis, although the proteases responsible for this processing are unknown (16, 22). If cysteine proteases independently mediate erythrocyte rupture, potential candidates include falcipain-2, which cleaves the erythrocyte cytoskeletal proteins ankyrin and band 4.1 in vitro (25, 26); falcipain-3, which is maximally expressed late in the life cycle (5); serine protease inhibitors of the proteasome, which cleave the erythrocyte cytoskeleton (27); and other putative cysteine proteases (32). Falcipain-1, which is expressed throughout the erythrocytic cycle and has been localized to merozoites (8), also may play a role in erythrocyte rupture and invasion, but our data indicate that it is not required for these processes. Falcipain-1 likely also plays a role in other plasmodial life cycle stages. The processing of gametocyte (33) and sporozoite (P. S. S. & Rosenthal, P. J.) proteins is inhibited by cysteine protease inhibitors, and genomic (34) and proteomic (35) surveys identified falcipain-1 in sporozoites.

Our data indicate that falcipain-1 is not essential in erythrocytic-stage malaria parasites. It clearly will be of interest to extend studies with falcipain-1 knockouts to other stages. Regarding the development of new drugs directed against essential processes in erythrocytic parasites, it appears that falcipain-2 and falcipain-3 are the major cysteine proteases involved in hemoglobinolysis, and that among the falcipains, they should be primary targets for drug discovery.

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