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Targeted disruption of *Plasmodium falciparum* cysteine protease, falcipain 1, reduces oocyst production, not erythrocytic stage growth

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Summary

Cysteine proteases are currently targets for drug development in a number of parasitic diseases, including malaria. In *Plasmodium falciparum*, the parasite responsible for the most virulent form of human malaria, there are four members of the cathepsin L-like family of cysteine proteases. Three of these (falcipains 2A, 2B and 3) are thought to be primarily involved in haemoglobin digestion, whereas falcipain 1 has recently been linked to erythrocyte invasion. Neither their expression nor their role in *P. falciparum* gametocytogenesis, which is required for malaria transmission, has been evaluated. In this study, RNA transcripts for the falcipain family members were identified as the parasite developed through all five stages of gametocytogenesis. Falcipain 1 transcript was upregulated in gametocytes, while levels of falcipain 2A/2B decreased in late-stage gametocytes and gametes. To evaluate the function of falcipain 1, the gene was disrupted, and clones from independent transformations were isolated. The asexual growth of the falcipain 1 minus clones was not overtly affected, and they produced morphologically normal gametocytes and gametes. However, when falcipain 1 minus parasites were fed to a mosquito, oocyst production was reduced by 70–90%, suggesting an important role for falcipain 1 during parasite development in the mosquito midgut.

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Introduction

Malaria caused by the protozoan parasite *Plasmodium falciparum* continues to be a global health problem. The development of drug resistance and the parasite's complex life cycle contribute significantly to the difficulties involved in controlling the disease. Each developmental stage of the parasite is distinct and may respond differently to interventions, such as drugs or vaccines. For example, many of the most commonly used malaria drugs target the asexual erythrocytic forms of the parasite that cause clinical symptoms, but are ineffective against the sexual forms that are required for transmission from one person to another. Recent evidence indicates that the antimalarial drug pyrimethamine is actually associated with an increase in circulating gametocytes (von Seidlein *et al*., 2001; Bousema *et al*., 2003).

Among the current targets of malaria drug development are cysteine proteases, which have been studied in *P. falciparum* asexual parasites for a number of years (Rosenthal *et al*., 2002). However, neither their expression in any other stage of the parasite's life cycle nor their role in malaria transmission has been studied. The first *P. falciparum* cysteine protease was cloned in 1992 and called falcipain (Rosenthal and Nelson, 1992). Subsequently, additional homologues have been identified, and the three genes have been named falcipain 1, 2 and 3 in the order in which they were identified (Shenai *et al*., 2000; Sijwali *et al*., 2001). Further analysis of the completed *P. falciparum* genome revealed a fourth member of the falcipain family, which is 96% identical at the nucleotide level and 93.4% identical at the amino acid level to falcipain 2 (Nielsen *et al*., 2003). The similarity between these two genes makes them very difficult to differentiate using hybridization techniques; consequently, previous work referring to falcipain 2 could be either or both genes. The original gene (PF11-0165) will be referred to as falcipain 2A, and the upstream homologue (PF11-0161) will be referred to as falcipain 2B. Proteomic data released with the completed genome indicated that peptides corresponding to falcipain 3 were identified in gametes, but there have been no other reports on the expression of these proteases in the sexual stages (Lasonder *et al*., 2002).

The functions of cysteine proteases in asexual *P. falciparum* parasites have been studied using specific inhibitors and localization studies, as well as the enzyme activity of isolated native and recombinant proteins. Cysteine protease-specific inhibitors have been reported to block haemoglobin digestion in the food vacuole, merozoite release and, most recently, merozoite invasion of erythrocytes (Rosenthal *et al*., 1988; Shenai *et al*., 2000; Salmon *et al*., 2001; Greenbaum *et al*., 2002; Malhotra *et al*., 2002; Wickham *et al*., 2003). Falcipain 2 and 3 are thought to be involved in haemoglobin digestion, based on their localization to the food vacuole and the observation that recombinant forms of both proteins degrade haemoglobin *in vitro* at low pH (Shenai *et al*., 2000; Sijwali *et al*., 2001). The cysteine protease involved in merozoite release has not been identified, although falcipain 2 has been shown to degrade red blood cell (RBC) cytoskeletal components ankryrin and band 4.1 *in vitro* (Hanspal *et al*., 2002; Dhawan *et al*., 2003). The inhibition of RBC invasion has been attributed to falcipain 1 based on timing of protease activity and the use of a selective inhibitor, YA29, identified using a library screening approach (Greenbaum *et al*., 2002).

The work reported here evaluates the expression of the falcipain family during sexual differentiation and uses targeted gene disruption to analyse the functional role of falcipain 1 during asexual and sexual stage development within the erythrocyte and through the parasite's transition to the mosquito. Gametocytogenesis and successful sporogonic development in the mosquito are required for malaria transmission from one person to another, and the identification of genes that are important during development in the mosquito could lead to additional malaria control strategies.

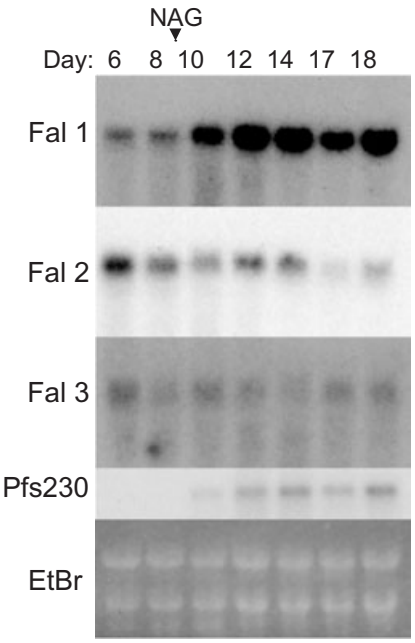

Fig. 1. Northern blot of RNA from asexual- and sexual-stage parasites. RNA was isolated from parasites obtained on the indicated day from a culture started on day 1 at 0.1% asexual parasitaemia. On days 6 and 8, the culture contained primarily asexual parasites. On day 9, 50 mM N-acetyl-D-glucosamine (NAG) was added to eliminate asexual parasites. The predominant stage in the cultures on days 10, 12, 14 and 17 was II, III, IV and V gametocytes respectively. On day 18, the mature stage V gametocytes were stimulated and, 1 h later, the gametes were isolated and used to obtain RNA. The first panel (Fal 1) is probed with random prime-labelled falcipain 1 bp 973–1776; the second panel (Fal 2), falcipain 2, PF11-0165 bp 34–146; the third panel (Fal 3), falcipain 3 bp 34–1496; the fourth panel (Pfs230), Pfs230 bp 64–301; and the fifth panel (EtBr) is the corresponding ethidium bromide-stained agarose gel.

Results

Northern analysis of RNA obtained during the transition of asexual parasites through gametogenesis revealed that falcipain 1 transcripts increased as stage III gametocytes were produced and remained high through gametogenesis (Fig. 1). Falcipain 2A/2B levels decreased in stage V gametocytes and remained low in gametes, while falcipain 3 levels remained constant. To determine the role of falcipain 1, it was targeted for disruption using transformation plasmid pDT.Tg23 containing the 5′ end of the falcipain gene, bp 25–612. Pyrimethamine-resistant parasites from two independent transformations that contained an integrated copy of the plasmid were cloned by limiting dilution (Fal 1-B and Fal 1-F). Southern blotting of *Kpn*I-digested genomic DNA from both clones confirmed chromosomal integration into the falcipain 1 gene, and Northern analysis demonstrated that the 3′ end of the falcipain gene (bp 904–1707), which contains the cysteine protease active site, was no longer expressed by either clone (Fig. 2B and C). *Kpn*I cuts the falcipain gene at bp 996, which is 3′ to the region of falcipain 1 that was inserted in the plasmid. After chromosomal integration, *Kpn*I digestion will release a 2.7 kb fragment that includes 1715 bp of pDT.Tg23 and bp 25–996 of the falcipain gene. The continued presence of the 5.2 kb band corresponding to the plasmid in the clones could result from multiple tandemly integrated copies of the plasmid or retained episomal plasmid. Both possibilities have been reported previously in *P. falciparum* (Kadekoppala *et al*., 2001; O'Donnell *et al*., 2001).

To demonstrate further that active protein was no longer expressed, ^{125}I-labelled DCG-04 and falcipain 1 peptide-specific polyclonal antibody were used (Fig. 3). DCG-04 was developed as a chemical probe specifically to identify active cysteine proteases of the papain family. The irreversible cysteine protease inhibitor E64 was used as a scaffold, and a tyrosine was added that can be iodinated without affecting the specificity of the compound (Greenbaum *et al*., 2000). ^{125}I-labelled DCG-04

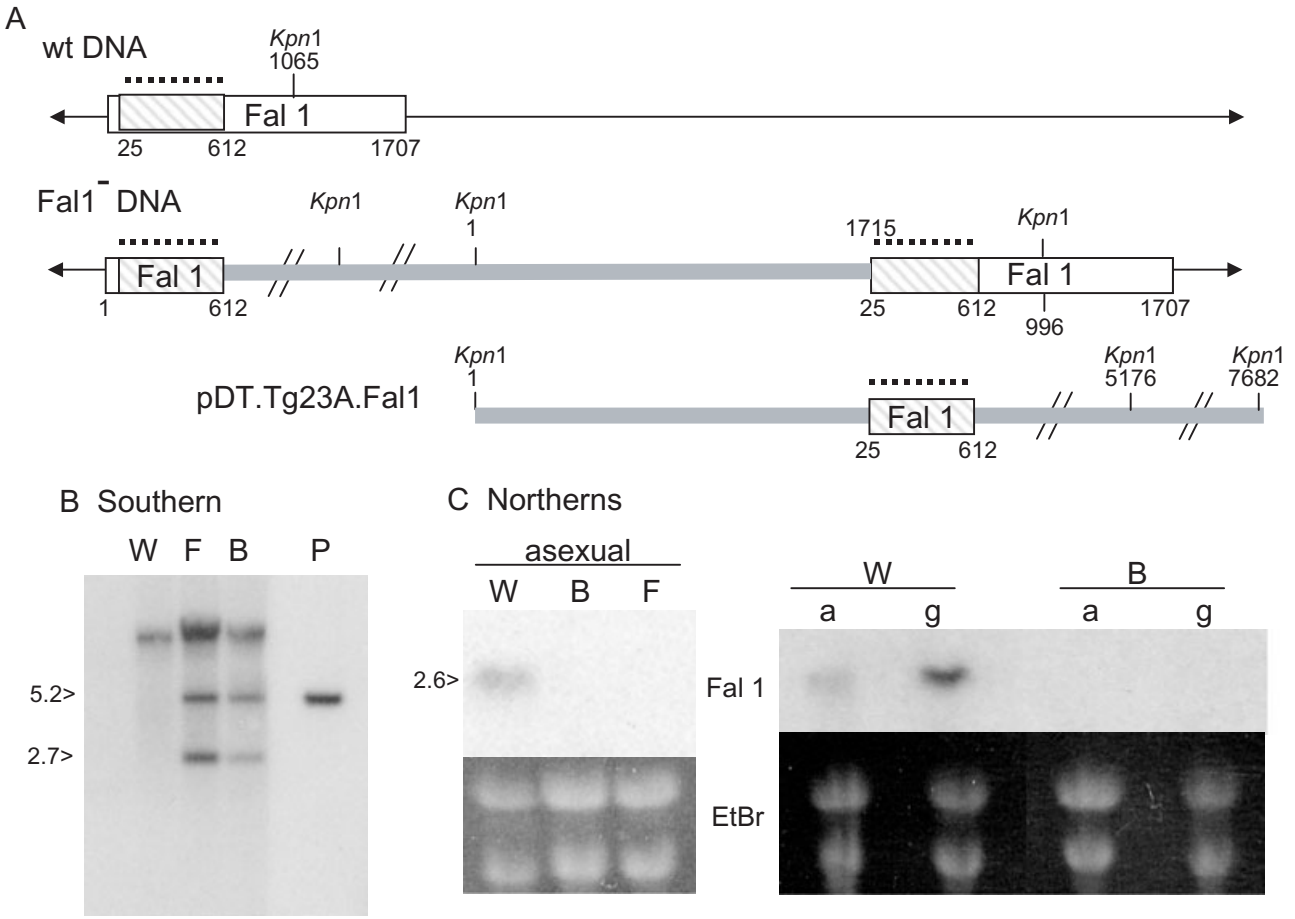

Fig. 2. Targeted disruption of the falcipain 1 gene.
A. Schematic of the falcipain 1 gene before (wt DNA) and after integration of plasmid pDT.Tg23.fal 1.25–612 (Fal1⁻ DNA) or the plasmid used for targeted disruption, pDT.Tg23.fal 1.25–612 (pDT.Tg23.fal 1). The falcipain 1 coding region is indicated by an open box, the hatched box is the region of the gene included in the disruption plasmid, and sequence from the plasmid backbone is indicated by a thick grey line. Numbers below the schematic designate the falcipain 1 gene, and numbers above the schematic designate the plasmid backbone. The location of the *Kpn*I restriction sites and the probe sequence (dotted line) used to evaluate integration are indicated.
B. Southern blot of *Kpn*I-digested genomic DNA isolated from wild-type 3D7 (W) and pDT.Tg23.fal 1.25–612 transformant clones F and B (F) and (B), as well as *Kpn*I-digested pDT.Tg23.fal 1.25–612 plasmid DNA (P). The blot was probed with falcipain 1 bp 25–612 (A, dotted line).
C. Northern blot of RNA isolated from asexual (a) or gametocyte (g) cultures of wild-type 3D7 parasites (W) and pDT.Tg23.fal 1.25–612 transformant clones F and B (F) and (B). The top (Fal 1) is probed with falcipain 1 bp 904–1707, and the bottom (EtBr) is the corresponding ethidium bromide-stained agarose gel as an RNA loading control.

has been shown to label the active site of papain-like cysteine proteases covalently and has been used to label falcipains in asexual parasites (Greenbaum *et al.*, 2002). The wild-type asexual pattern of ^{125}I-DCG-04 labelling obtained was the same as that reported previously (Greenbaum *et al.*, 2002). Asexual parasites that had been treated with 0.1% saponin to remove the RBCs were lysed by freeze–thawing and labelled with [^{125}I]-DCG-04 at pH 5.5 in the presence of 0.1% IGEPAL (which replaced NP-40). The soluble fraction was isolated by centrifugation, and the pellet was solubilized in 1.0% SDS. The migration of the predominant ^{125}I-DCG-04-labelled band in the soluble fraction (S) was consistent with the sizes of active falcipain 2A/2B and 3, ≈31 kDa, and will be referred to as fal 2/3. The predominant ^{125}I-DCG-04-labelled band in the SDS-extracted fraction (D) was consistent with the size, ≈26 kDa, of active falcipain 1 and will be referred to as fal 1. Consistent with this designation, the falcipain 1 band was the only band that was absent in the fal 1 disruptant clones (Fig. 3, lane F1⁻, D). When the same fractions were probed with fal 1 antibody, the 26 kDa band in the wild-type SDS-extracted fraction disappeared (Fig. 3). A larger band, which migrates slightly faster than the ^{125}I-labelled fal 2/3 band, was observed in the soluble fraction of both the clone and wild-type parasites. The growth rate of the asexual parasites and induction of gametocytogenesis is the same in wild-type (WT), parasites transformed with the pDT.Tg23 vector alone (control transformants, CT) and fal 1⁻ clones B and F, as is

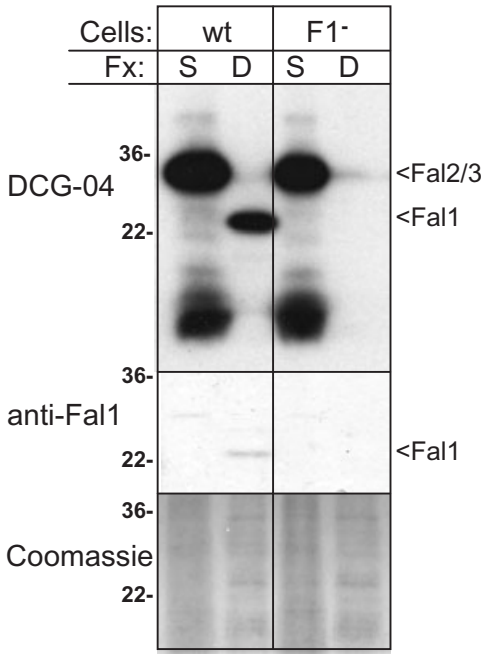

Fig. 3. 125I-DCG-04 labelling of asexual parasites. Asexual wild-type (WT) or fal 1-B ($F1^-$) parasites were labelled with [^{125}I]-DCG-04 at pH 5.5 and the 0.1% IGEPAL-soluble (S) and -insoluble (D) fractions obtained were size fractionated and transferred to nitrocellulose. The top (DCG-04) is the autoradiograph of the blot, the middle (anti-Fal1) is a Western blot probed with falcipain 1 peptide-specific polyclonal antibody, and the bottom (Coomassie) is the Coomassie stain of the acrylamide gel as a loading control.

the progression through the five stages of gametocytogenesis (Fig. 4). The stage V gametocytes produced by the fal 1^- clones are morphologically normal, can be stimulated to round up and emerge from the RBCs with xanthurenic acid, and the males exflagellate. However, when fed to mosquitoes, oocyst production is reduced 70–90% (Table 1). Both the number of fed mosquitoes that were infected and the number of oocysts per midgut are decreased.

^{125}I-DCG-04 labelling at both pH 5.5 and 7.5 confirmed the presence of active falcipain 1 in both gametocytes and gametes produced by the control transformed clones and its absence in the fal 1^- clones (Fig. 5A). The disruption of falcipain 1 also does not alter the labelling pattern of the other bands, suggesting that the expression of another cysteine protease is not upregulated in the absence of falcipain 1. The ^{125}I-DCG-04 labelling of falcipain 1 at both pHs indicates that falcipain 1 is active over a wide pH range; however, its solubility profile changes depending on the pH. At pH 5.5, either 0.5% Triton or 1% SDS is required for solubilization, whereas at pH 7.5 a fraction of the labelled falcipain 1 is soluble. At pH 7.5, a number of additional bands >30 kDa are also labelled. These could be cysteine proteases that are partially processed or cysteine proteases with neutral pH optima, perhaps located in the cytoplasm or neutral

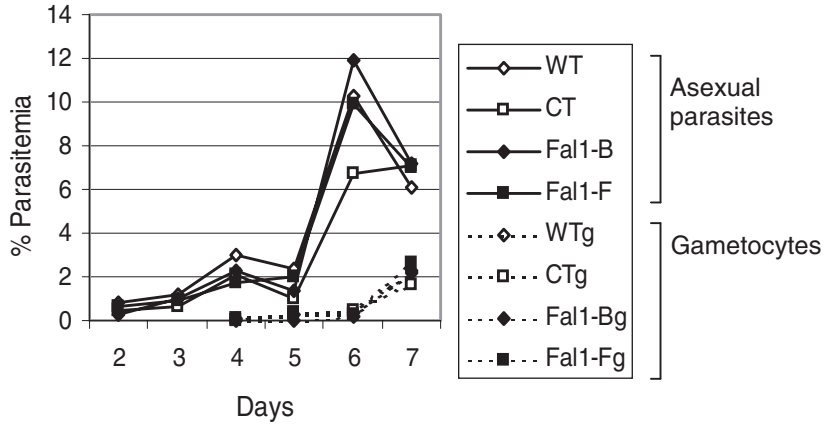

Fig. 4. Asexual growth and induction of gametocytogenesis. Cultures of wild-type 3D7 (WT, open diamonds), 3D7 transformed with pDT.Tg23 alone, control transformants (CT, open squares) or pDT.Tg23.fal 1.25–612 transformant clones F and B (Fal 1-B, filled diamonds; and Fal 1-F, filled squares) were started on day 1 at 0.1% asexual parasitaemia, and daily Giemsa-stained smears were made to monitor the asexual (solid line) and sexual (dotted line) parasitaemia until the onset of gametocytogenesis.

Table 1. Mosquito feeds.

Parasite	Oocyst no. Average (range)	% Oocyst reduction	Infected/ dissected	% Infectivity
Control transformant	5.72 (0–17)	0.0	16/18	88.8
Fal 1^-	1.67[a] (0–7)	70.8	8/12	66.6
Fal 1^-	0.45[a] (0–1)	92.1	5/18	27.8

a. Significant decrease in oocyst number per midgut ($P \leq 0.005$) by Kruskal–Wallis analysis.
The results are from mosquito feeds of mature gametocyte cultures of the indicated clones. The control transformant clone was isolated from parasites transfected with the pDT.Tg23 vector alone. The average and range of the number of oocysts per midgut, the percentage reduction in oocyst from the control transformants (% oocyst reduction), as well as the number of dissected mosquitoes that were infected (>one oocyst per midgut) and the percentage of the dissected mosquitoes that were infected (% infection) are listed. The groups were compared using a Kruskal–Wallis test.

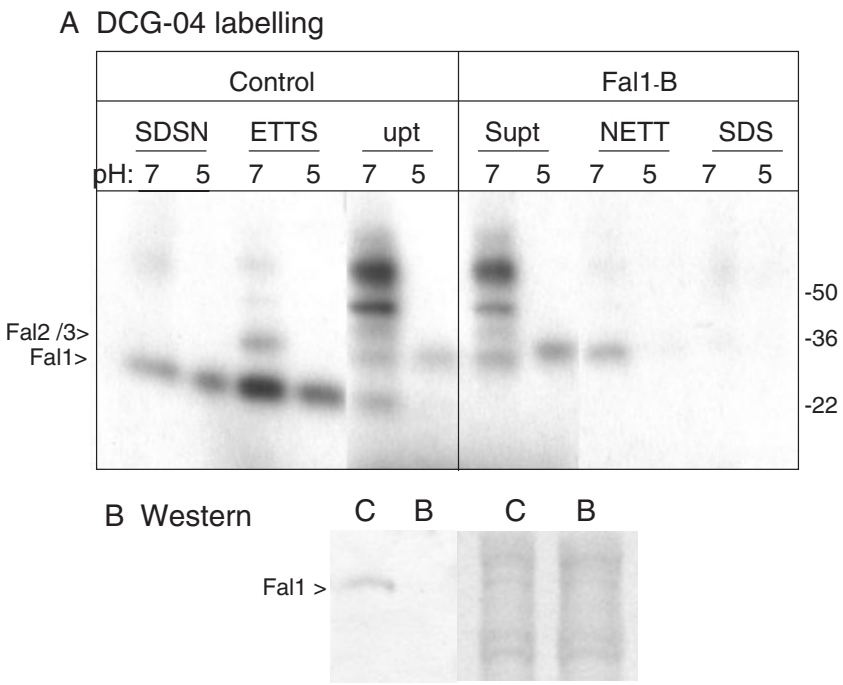

Fig. 5. Falcipain 1 expression in stage V gametocytes
A. ^{125}I-DCG-04 labelling of stage V gametocytes. Control transformant (Control) or fal 1-B (Fal 1$^-$ B) parasites were labelled with [^{125}I]-DCG-04 at pH 5.5 (5) or pH 7.5 (7), and the 0.1% IGEPAL-soluble supernatants (Supt) were isolated. The insoluble pellets were extracted first in 0.5% Triton X-100 (NETT) and then 1% SDS (SDS). Equivalent amounts of all the fractions were size fractionated, transferred to nitrocellulose and used for autoradiography. The migration of the molecular weight markers is indicated on the right, and the location of the fal2/3 and fal1 bands is indicated on the left.
B. Western blot of stage V gametocytes from Control transformant (C) or fal 1-B (B) cultures. The left panel is probed with falcipain 1 peptide-specific polyclonal antibody, and the right panel is stained with Ponceau S as a loading control.

vesicles. As mention above, the pattern of these bands is not altered in the fal 1$^-$ clones, indicating that they are not active forms of partially or unprocessed falcipain 1. Furthermore, the absence of falcipain 1 in gametocytes produced from fal 1$^-$ clones was confirmed by both Northern and Western blotting (Figs 2C and 5B).

Discussion

This work is the first to report the targeted disruption of a cysteine protease in *P. falciparum* and demonstrates a significant role for falcipain 1 in oocyst production, but not an essential role in asexual or gametocyte/gamete development. Consistent with the important role of falcipain 1 in the development of the parasite in the midgut is the increased level of transcript observed during gametocytogenesis. Once the gametocyte is taken up by the mosquito, it emerges from the RBCs as an extracellular gamete and no longer has to rely on the digestion of intact haemoglobin, which has been suggested as the major function of falcipain 2 and 3 (Shenai *et al*., 2000; Sijwali *et al*., 2001). It is possible that, in the mosquito midgut, falcipain 1 is required for nutrient digestion. However, the broad pH range of falcipain 1 ^{125}I-DCG-04 labelling opens the possibility that its function is not restricted to an acidic environment such as the food vacuole. The distinct solubility profile of falcipain 1 also suggests that its subcellular location is different from that of falcipain 2 and 3. Unfortunately, direct subcellular localization was not possible by IFA, because the anti-fal 1 antibodies were not reactive with methanol, acetone or formaldehyde-fixed wild-type gametocyte preparations. Falcipain 1 could be directly involved in the actual transition from gamete to oocyst by activating proteins by proteolytic processing or, if secreted, it could degrade the peritrophic matrix or midgut endothelium facilitating the migration of the ookinete. The transition from gamete to oocyst is complex, requiring fertilization, ookinete differentiation and migration through the peritrophic matrix and midgut endothelium. *In vitro* methods have not yet been developed to produce *P. falciparum* ookinetes and oocysts; therefore, further studies evaluating the specific role of falcipain 1 in oocyst production will have to be evaluated in mosquito midgut preparations.

A recent report, based on the specificity of cysteine protease inhibitor YA29 for falcipain1, has indicated that falcipain 1 is important for RBC invasion by the merozoite (Greenbaum *et al*., 2002). Our work clearly demonstrates that falcipain 1 minus asexual parasites grow normally, indicating that falcipain 1 is not an absolute requirement for RBC invasion. It is very possible that there are redundant pathways for such a critical step in parasite development and that the knock-out process could have selected a parasite clone that has upregulated another gene/s to compensate for the loss of falcipain 1. Alternatively, the inhibitor, YA29, may not be absolutely selective and could also inhibit redundant pathways. Another option is that the generation of fal 1 disruptant clones selected for an alternative invasion pathway as has been shown to happen when EBA175

is disrupted (Reed *et al*., 2000; Duraisingh *et al*., 2003). The basic process of generating and cloning transformed *P. falciparum* parasites requires both drug resistance and multiple rounds of successful asexual replication, which includes RBC invasion. Therefore, in this case, only parasites that retain the ability to invade RBCs in the absence of the falcipain 1 would have been selected. In contrast, in the study by Greenbaum *et al*. (2002), which demonstrated a dramatic block in invasion, wild-type parasites were only grown in the presence of YA29 for the final 8–10 h of a single ≈48 h life cycle. In this brief time period, the parasites may not be able to compensate for the rapid loss of falcipain 1 activity.

The identification of an important role for falcipain 1 in oocyst production and the demonstration of the expression of additional cysteine proteases during sexual differentiation provide other points in the parasite life cycle to be considered in malaria drug design. The specific molecular mechanisms involved and the role of other cysteine proteases remain to be elucidated, but this work clearly indicates the importance of these proteins throughout the life cycle. The ability to block multiple points in the parasite's life cycle could lead to an increase in effectiveness and longevity of intervention strategies targeting these proteins.

Experimental procedures

Plasmodium falciparum *parasites*

Plasmodium falciparum parasites were maintained in culture and gametocytogenesis was induced as described by Ifediba and Vanderberg (1981). Parasite cultures were harvested, washed in phosphate-buffered saline (PBS) and then isolated by centrifugation after incubation with 0.1% saponin–PBS for 5 min at room temperature to obtain asexual parasites or 0.015% saponin–PBS for 10 min at 37°C to obtain gametocytes.

Transformation

Falcipain 1 bp 25–612 was amplified by polymerase chain reaction (PCR) using the following primers: 5′-TAAT ACT AGT GAA CTT GCA TTT GCT AGA CCA and 5′-TAAT CCG CGG CAT ACC TAT TTC CAA AGC TCC. The PCR product was digested with *Spe*I and *Sac*II, then ligated into pDT.Tg23 (provided by Y. Wu and T. Wellems) that had been *Spe*I and *Sac*II digested and treated with bacterial alkaline phosphatase (Wu *et al*., 1996). The plasmid obtained, pDT.Tg23.fal 1.25–612, was screened for insert by restriction analysis and automated DNA sequencing (University of Chicago). pDT.Tg23.fal 1.25–612 or pDT.Tg23 (100 μg each) was used to transform *P. falciparum* (strain 3D7) infected RBCs (5 ml culture of 5–6% rings) as described by Eksi *et al*. (2002). To select for parasites with chromosomal integration of the plasmid, drug pressure was released for 3 weeks, then reapplied at a concentration of 500 ng ml^{-1}. The pyrimethamine-resistant parasites obtained were screened for plasmid integration by restriction analysis and cloned by limiting dilution. Clones from two independent transformations with pDT.Tg23.fal 1.25–612 were isolated and analysed further (clones B and F).

DNA and RNA analysis

DNA was isolated by phenol–chloroform extraction from parasites that had been pretreated with 0.2% saponin and then lysed in 10 mM NaCl, 2% sodium dodecyl sulphate (SDS), 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 20 μg ml^{-1} proteinase K (Wu *et al*., 1996). DNA was analysed for plasmid integration by Southern blot after standard horizontal electrophoresis. RNA for the Northern blot was isolated using Trizol (Invitrogen) from parasite cultures that had been washed three times in 4°C PBS before incubation in 0.01% saponin–PBS at room temperature for 5 min, then washed three times in 4°C PBS.

^{125}I-DCG-04 labelling

DCG-04 was iodinated using iodogen-coated beads (Pierce) as described by Greenbaum *et al*. (2002). Samples of 50 mM phosphate buffer, pH 7.4 (35 μl), 1 mCi of ^{125}I and 5 mM DCG-04–50% ethanol (12.5 μl) were incubated with an iodogen-coated bead for 15 min at room temperature. An additional 100 μl of 50 mM phosphate buffer, pH 7.4, was then added, and the entire reaction was applied to a SepPak C18 cartridge (Waters) that had been wetted with acetonitrile (5 ml) and then equilibrated with 50 mM phosphate buffer, pH 7.4 (10 ml). The SepPak 18 cartridge containing the reaction mixture was washed with 50 mM phosphate buffer, pH 7.4 (30 ml), then [^{125}I]-DGC-04 was eluted with acetonitrile in 1 ml fractions and used to label parasites.

Frozen pellets of saponin-treated asexual parasites or gametocytes (2×10^7 cells) were resuspended in 40 μl of 50 mM sodium acetate buffer, pH 5.5, 1 mM dithiothreitol (DTT), 0.1% (octylphenoxy)polyethoxyethanol (IGEPAL CA-630, which replaced NP-40), and 4 μl of [^{125}I]-DGC-04 was added. After a 1 h incubation at room temperature, the soluble fraction was isolated by centrifugation, and the pellet was further extracted in 40 μl of 1.0% SDS–50 mM Tris, pH 6.8, or NETT (150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 50 mM Tris, pH 8.0) followed by centrifugation to isolate the detergent soluble fraction.

Western blots

SDS sample buffer was added to the indicated parasite fraction to obtain a final concentration of 1% SDS, 10% glycerol, 5% β-mercaptoethanol, 125 mM Tris-Cl (pH 6.8). The sample was heated to 95°C for 5 min, size fractionated on a 4–20% polyacrylamide gel (Invitrogen), then transferred to nitrocellulose. The nitrocellulose blot was incubated with anti-falcipain 1 peptide (VPEILDYREKGIVH)-specific antiserum that was generated by Covance using rabbits injected intra-

peritoneally with peptide–KLH conjugate. The antibody was purified by affinity chromatography. Briefly, the peptide was immobilized on iodoacetyl-modified agarose, and the antibody was eluted using 100 mM glycine (pH 2.5). The antibody-labelled bands were visualized with alkaline phosphatase-conjugated secondary antibodies (Sigma; 1:2000), 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT; Invitrogen), and the ^{125}I-DGC-04-labelled band were visualized by autoradiography.

Mosquito feed

A 0.6 ml aliquot of a *P. falciparum* culture containing mature gametocytes was pelleted on to 120 μl of packed erythrocytes. The supernatant was removed and replaced by 120 μl of normal human serum containing active complement. The mixture was introduced into a water-jacketed membrane feeder maintained at 37°C, and *Anopheles stephensi* mosquitoes were allowed to gorge for 10 min, then grown for 7 more days at 26°C and >80% humidity. The midguts were then dissected and stained in 0.2% mercurochrome to visualize the *P. falciparum* oocysts.

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