

# Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*

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## Summary

Malaria remains a public health problem of enormous magnitude, affecting over 500 million people every year. Lack of success in the past in the development of new drug/vaccines has mainly been attributed to poor understanding of the functions of different parasite proteins. Recently, RNA interference (RNAi) has emerged as a simple and incisive technique to study gene functions in a variety of organisms. In this study, we report the results of RNAi by double-stranded RNA of cysteine protease genes (*falcipain-1* and *-2*) in the malaria parasite, *Plasmodium falciparum*. Using RNAi directed towards falcipain genes, we demonstrate that blocking the expression of these genes results in severe morphological abnormalities in parasites, inhibition of parasite growth *in vitro* and substantial accumulation of haemoglobin in the parasite. The inhibitory effects produced by falcipain double-stranded (ds)RNAs are reminiscent of the effects observed upon administering E-64, a cysteine protease inhibitor. The parasites treated with falcipain's dsRNAs also show marked reduction in the levels of corresponding endogenous *falcipain* mRNAs. We also demonstrate that dsRNAs of falcipains are broken into short interference RNAs  $\approx$  25 nucleotides in size, a characteristic of RNAi, which in turn activates sequence-specific nuclease activity in the malaria parasites. These results thus provide more evidence for the existence of RNAi in *P. falciparum* and also suggest possibilities for using RNAi as an effective tool to determine the functions of the genes

identified from the *P. falciparum* genome sequencing project.

## Introduction

In the fight against malaria, there is an urgent need to develop new antimalarials and an effective vaccine because of widespread resistance to common antimalarials (Ridley, 2002). The progress in the development of effective antimalarials/vaccine has been slow because of a poor understanding of the functions of different parasite proteins (Cowman *et al.*, 2000). One of the direct ways to determine the biological function of a protein is to examine the phenotype of the organism that contains mutations in the coding gene or to knock out the gene by homologous recombination. In recent years, methodologies have been developed that permit transfection of *Plasmodium falciparum*, *Plasmodium berghei* and *Plasmodium knowlesi*, as well as the deletion of specific parasite genes in order to study their function (Waters *et al.*, 1997). However, malaria transfection studies, together with previously employed biochemical approaches, have provided limited insight into our understanding of the molecular basis of parasite infection (Cowman *et al.*, 2000). With the completion of the malaria genome project, there is an urgent need for additional methods for assessing gene functions in the malaria parasite.

Recently, RNA interference (RNAi) has emerged as a powerful method for studying gene functions in a wide range of organisms, both unicellular organisms such as *Trypanosoma brucei* and *Plasmodium falciparum* (Ngo *et al.*, 1998; McRobert and McConkey, 2002) and multicellular organisms such as *Caenorhabditis elegans*, *Drosophila*, *Planaria*, *Hydra*, zebrafish and mouse (Cogoni and Maino, 2000). The term RNAi refers to the sequence-specific degradation of mRNA by its homologous double-stranded (ds)RNA (Fire, 1999). Although RNAi was first demonstrated in nematodes (Fire *et al.*, 1998), similar phenomena, called post-transcriptional gene silencing (PTGS) in plants (Baulcombe, 1999) and 'quelling' in fungi (Cogoni *et al.*, 1996), have been known for many years. Recently, RNAi was applied successfully to study the functions of a large number of genes on a

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genomic scale in *C. elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000; Maeda *et al.*, 2001).

Given the fact that gene knock-out experiments in *P. falciparum* are difficult to carry out and RNAi has been used to study gene functions in variety of organisms (Zamore *et al.*, 2000), we explored the possibility of using this technique in *P. falciparum*. We used dsRNAs of the two cysteine protease genes of *P. falciparum* to evaluate their metabolic roles in the erythrocytic stages of the parasite. Degradation of haemoglobin is a prerequisite for the establishment and multiplication of the parasite. A series of experiments on haemoglobin degradation pathways in *P. falciparum* led to the identification of three cysteine proteases (also known as falcipain 1, falcipain 2 and falcipain 3), 10 aspartic proteases (called plasmepsins) and metalloproteases involved in haemoglobin degradation (Coombs *et al.*, 2001; Eggleston *et al.*, 1999; Sijwali *et al.*, 2001). However, the exact roles of these *Plasmodium* proteases in the haemoglobin degradation pathway are not clearly understood. To understand the precise roles of falcipains in the haemoglobin degradation pathway, we carried out silencing of two *falcipain* genes by their respective dsRNAs. These experiments revealed that the treatment of parasites by *falcipain*-1 and -2 dsRNAs not only results in marked reduction in the levels of cognate cellular messenger RNA but also brings about several morphological and biochemical changes, which are reminiscent of changes induced by other specific inhibitors of cysteine proteases. Detailed analysis of the mode of RNAi action revealed that these effects are mediated by the formation of an  $\approx 25$  nucleotide (nt) RNA species, a feature characteristic of all PTGS phenomena. These results have important implications for carrying out large-scale functional genomic studies in *P. falciparum*.

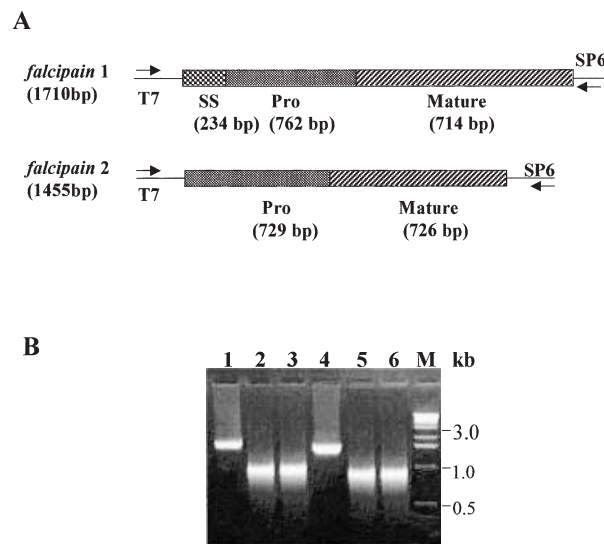
## Results

### RNAi functions in *P. falciparum*

The hallmarks of RNAi are its specificity, simplicity and the ease by which RNAi can be applied to an organism. RNAi has been induced in most organisms by microinjections (Fjose *et al.*, 2001). However, in *C. elegans*, large-scale gene silencing by RNAi was carried out successfully simply by soaking worms in dsRNA containing solution or by feeding worms with *Escherichia coli* that express dsRNA (Maeda *et al.*, 2001). In *Drosophila* S2 cell lines, RNAi was used for dissecting signal transduction pathways just by adding dsRNA to the culture medium (Clemens *et al.*, 2000). Based on these observations, we decided to study RNAi in *P. falciparum* by adding dsRNAs to the culture medium using the protocol described by Clemens *et al.* (2000). We have successfully carried out gene silencing of aminopeptidase-N gene in *Spodoptera*

*frugiperda* 21 (Sf21) cell lines using the same protocol (unpublished results). All the dsRNAs used in the present study were analysed on 1% agarose gel to make sure that these were predominantly double stranded (Fig. 1B). As *P. falciparum* is an intracellular parasite, at the beginning of our analysis of RNAi, we studied the uptake of  $^{32}\text{P}$ -labelled dsRNAs of *falcipains* by malaria parasites. To do this, synchronized *P. falciparum* cultures were incubated with 10 nM  $^{32}\text{P}$ -labelled dsRNA from either of the *falcipains*. After 24 h of incubation, parasitized erythrocytes were collected by centrifugation, lysed with saponin and washed three times to obtain a pure parasite pellet. Levels of radioactivity were measured in different fractions of parasitized red blood cells (RBCs). Approximately 0.1–0.15% of the input labelled dsRNAs were found to be associated with the purified parasite pellets, suggesting that dsRNAs were taken up by the parasite. This result was in line with the earlier observations by Rapaport *et al.* (1992), who showed uptake of labelled oligodeoxynucleotides by *P. falciparum*-infected erythrocytes. Uptake of antisense oligodeoxynucleotides (ODNs) from the culture medium and their inhibitory effects have also been reported in *P. falciparum* (Barker *et al.*, 1996).

To determine the effects of *falcipain* dsRNAs on *P. falciparum*, synchronized parasites at late ring stages were



**Fig. 1.** A. Schematic representation of the two *falcipain* genes of *P. falciparum* used to produce single-strand RNA and dsRNA *in vitro* (SS, signal sequence; Pro, pro region; T7/SP6, promoter sequences). B. Agarose gel electrophoresis of ssRNAs and dsRNAs of *falcipain*-1 and -2. To prepare dsRNA, antisense RNAs (asRNAs) and sense RNAs (ssRNAs) were synthesized using T7 and SP6 polymerases. ssRNA and asRNA from each of the *falcipains* were mixed, incubated at 65°C and cooled slowly to obtain their corresponding dsRNAs. Lane 1, *falcipain*-1 dsRNA; lane 2, *falcipain*-1 ssRNA; lane 3, *falcipain*-1 asRNA; lane 4, *falcipain*-2 dsRNA; lane 5, *falcipain*-2 ssRNA; lane 6, *falcipain*-2 asRNA.

**Table 1.** Effects of Falcipain dsRNAs and E-64 on the morphology and viability of cultured parasites.

Treatment	Concentration	Food vacuole abnormality <sup>a</sup>	Percentage ring parasites
Falcipain-1 dsRNA	10 µg ml <sup>-1</sup> (9.0 nM)	+	82
	25 µg ml <sup>-1</sup> (23.0 nM)	++	60
	50 µg ml <sup>-1</sup> (45.5 nM)	++	65
Falcipain-2 dsRNA	10 µg ml <sup>-1</sup> (11.0 nM)	+	90
	25 µg ml <sup>-1</sup> (27.0 nM)	++	70
	50 µg ml <sup>-1</sup> (50.0 nM)	++	77
Falcipain-1 and -2 dsRNAs	25 µg ml <sup>-1</sup> each	+++	35
	50 µg ml <sup>-1</sup> each	++++	30
Control (DEPC water)	—	—	100
E-64	100 mM	+++++	0
Aminopeptidase-N ( <i>Spodoptera litura</i> ) dsRNA	50 µg ml <sup>-1</sup>	—	99

a. Percent abnormal parasites: —, 0%; +≤, 10%; ++≤, 30%; +++≤, 40%; ++++≤, 60%; +++++≤, 75%

incubated with dsRNA for each of the two falcipains separately, as well as in combination, at three different concentrations of 10 µg ml<sup>-1</sup>, 25 µg ml<sup>-1</sup> and 50 µg ml<sup>-1</sup>, corresponding to approximate final concentrations of 10 nM, 25 nM and 50 nM respectively. Inclusion of dsRNAs in the parasite culture caused distinct morphological changes, the most notable of which was the abnormally swollen food vacuoles that contained an accumulation of malaria pigment (Fig. 2A). These effects were similar to that seen upon incubation of parasites with a well-established cysteine protease inhibitor, E-64 (Rosenthal, 1995). As shown in Table 1, at a 10 µg ml<sup>-1</sup> concentration of each of these dsRNAs, morphological effects seen on the cultured parasites were marginal. However, at 25 µg ml<sup>-1</sup> and 50 µg ml<sup>-1</sup> concentrations of dsRNA of *falcipains*-1 or -2, up to 30% of parasites developed food vacuole abnormalities. Significantly, when dsRNAs of both *falcipains* were included at the above concentrations, about 60% of the parasites showed food vacuole abnormalities. Addition of the two dsRNAs together to the parasite culture also reduced the formation of new rings as well as total parasitaemia. In contrast, addition of dsRNA from aminopeptidase-N (50 µg ml<sup>-1</sup>) of insect origin, used as a control in the present study, had no effect on either parasite morphology or its development.

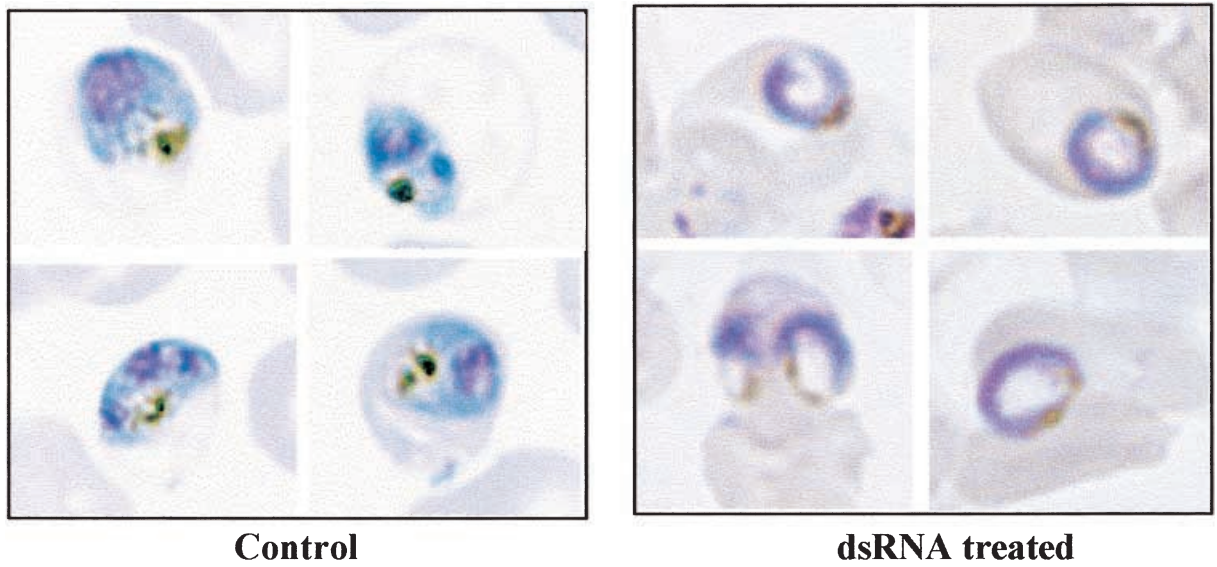
We confirmed further the inhibitory effects of *falcipain* dsRNAs on *P. falciparum* by an assay that uses [<sup>3</sup>H]-hypoxanthine uptake as a measure of parasite growth (Fig. 2B). In this assay, the aminopeptidase-N dsRNA was used as a negative control, whereas E-64 was used as a positive control. At a 50 µg ml<sup>-1</sup> concentration of individual *falcipain* dsRNA, there was a 25–35% reduction in the uptake of [<sup>3</sup>H]-hypoxanthine. However, when the two *falcipain* dsRNAs were combined, the reduction in the uptake of [<sup>3</sup>H]-hypoxanthine was enhanced to the level of ≈ 65%. In comparison, E-64 at 50 µM concentration almost completely inhibited the uptake of [<sup>3</sup>H]-hypoxanthine, whereas aminopeptidase-N dsRNA at this concentration

had no effect at all (Fig. 2B). These results were thus consistent with earlier observations, which showed that various inhibitors of cysteine proteases affect parasite metabolism and development.

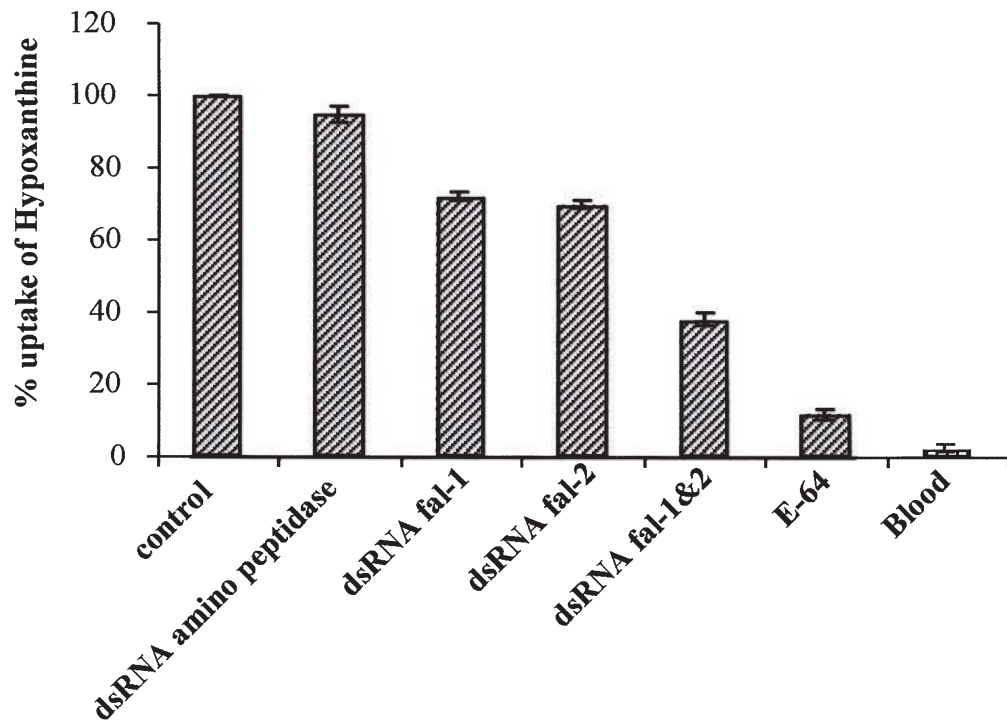
#### *Inhibition of haemoglobin degradation by falcipain dsRNAs in P. falciparum parasites*

Cysteine protease activities have mainly been identified in the extracts of trophozoites, an erythrocytic parasite stage during which most haemoglobin degradation occurs and most antimalarial drugs have been shown to act (Rosenthal, 1995). Various studies using either native falcipains or recombinant falcipains have demonstrated a central role for the falcipains in the haemoglobin degradation pathway (Sijwali *et al.*, 2001). It has been suggested that morphological abnormalities displayed by malaria parasites after treatment with different cysteine protease inhibitors result from inhibition of haemoglobin degradation (Rosenthal, 1995). To investigate whether the observed morphological abnormalities in *falcipain* dsRNA-treated parasites were indeed caused by a blockage in haemoglobin degradation, parasite cultures were treated with dsRNA from the two *falcipains*, separately or together, at 50 µg ml<sup>-1</sup> concentration, and the parasite proteins were subsequently analysed by SDS-PAGE. Incubation of parasites with dsRNAs for each of the *falcipains* caused the accumulation of large quantities of undegraded haemoglobin in these parasites (Fig. 3A, lanes 2–4). E-64, a general cysteine protease inhibitor, was used as a positive control (Fig. 3A, lane 6). In the control parasite culture containing no inhibitor, a minimum level of undegraded haemoglobin was observed (Fig. 3A, lane 1). The block in haemoglobin degradation produced by each *falcipain* dsRNA indicated that these dsRNAs were able to effect the functioning of these genes, i.e. these dsRNAs were able to induce specific gene silencing.

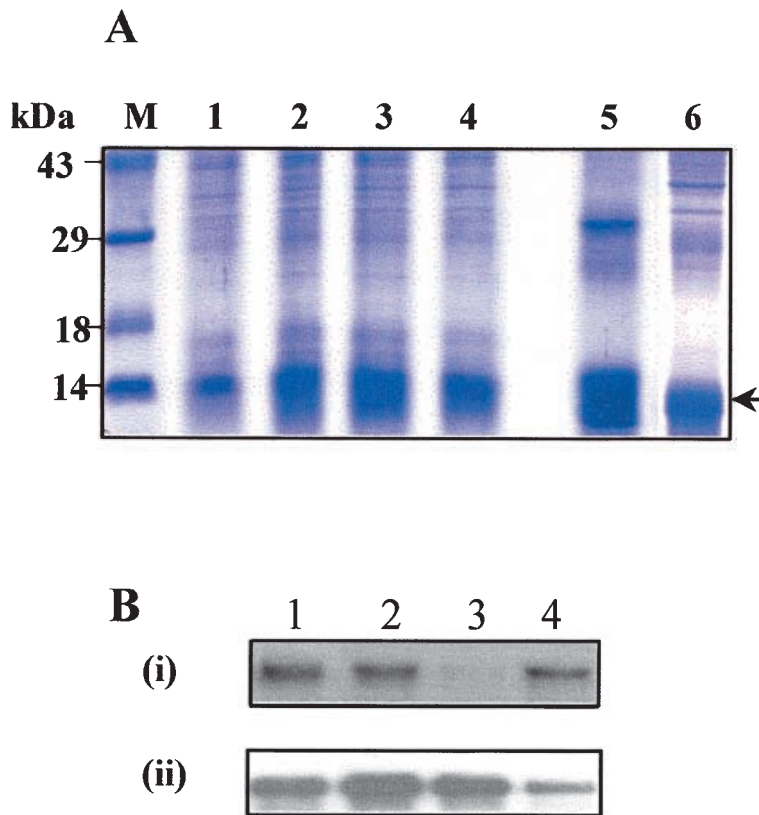
**A**



**B**



**Fig. 2.** A. Effects of *falcipain* dsRNA treatment on *P. falciparum* parasite morphology; note the abnormally swollen food vacuoles with malaria pigment in the treated parasites. B. Inhibition of [<sup>3</sup>H]-hypoxanthine uptake in cultured parasites by Falcipain dsRNAs. Microwell cultures of ring-stage parasites were incubated with Falcipain dsRNAs for 24 h. Approximately 1 μCi of [<sup>3</sup>H]-hypoxanthine was added to each well, and cultures were maintained for an additional 24 h. The cells were harvested, and [<sup>3</sup>H]-hypoxanthine was quantified using a scintillation counter. Aminopeptidase-N (*Spodoptera litura*) dsRNA was used as a control.



**Fig. 3.** A. Block in haemoglobin hydrolysis in *P. falciparum* parasites after treatment with *falcipain* dsRNAs. Cultured parasites were incubated for 48 h with: lane 1, water (control); lane 2, dsRNAs from *falcipains-1* and *-2*; lane 3, dsRNA from *falcipain-1*; lane 4, dsRNA from *falcipain-2*; lane 6, E64. Then, parasitized erythrocytes were lysed with saponin, and parasites were washed extensively to remove erythrocyte cytosolic components. Parasite pellets were solubilized in reducing SDS-PAGE sample buffer, and parasite proteins were resolved on 10% SDS-PAGE. Haemoglobin protein was run as standard (lane 5), undegraded globin appeared as a dimer at  $\approx 16$  kDa. Lane M, molecular weight markers.

B. Northern blot analyses showing effects of *falcipain* dsRNA treatment on levels of endogenous RNA transcripts. Total RNA was extracted from control parasites (lane 1), aminopeptidase dsRNA-treated parasites (lane 2), *falcipain-1* dsRNA-treated parasites (lane 3) and *falcipain-2* dsRNA-treated parasites (lane 4) and separated on 1.2% agarose gel for Northern blotting. Blots were hybridized with random prime-labelled *falcipain-1* DNA probe (i) and *falcipain-2* DNA probe (ii).

#### *Falcipain* dsRNAs induce degradation of homologous messenger RNA

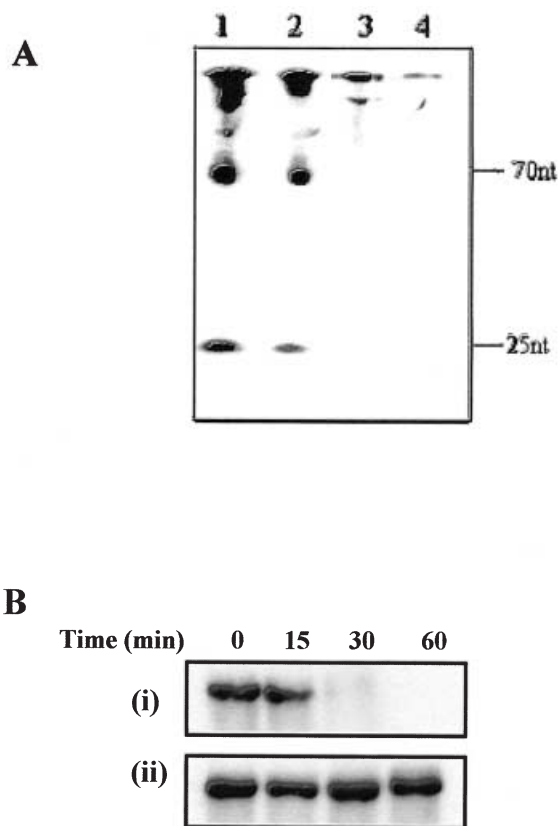
To determine whether morphological and biochemical effects induced by *falcipain* dsRNAs resulted from RNAi, we examined the fate of *falcipain* mRNAs in dsRNA-treated parasite cultures by Northern blot analysis. Parasites treated with the *falcipain-1* dsRNA showed diminished endogenous *falcipain-1* mRNA compared with the untreated, *falcipain-2* and *aminopeptidase-N* dsRNA-treated parasites [Fig. 3B(i)]. Similarly, treatment of parasites with *falcipain-2* dsRNA caused marked reduction in *falcipain-2* mRNA compared with control parasites, whereas the level of *falcipain-1* mRNA in *falcipain-2* dsRNA-treated parasites was comparable with that in control parasites [Fig. 3B(ii)]. Overall, in *falcipain-1* or *falcipain-2* dsRNA-treated parasites, there was a significant reduction in the levels of cognate mRNAs compared with their levels in untreated parasites. These results indicated that inhibitory effects seen in parasite cultures after treatment with *falcipain* dsRNAs are caused specifically by RNAi.

#### Dissection of the mechanism of RNAi in *P. falciparum*

A striking paradigm to emerge from the study of RNAi/PTGS in plants and animals is that gene silencing in these

organisms occurs as a result of the generation of small RNA called guide RNA or small interfering RNA (siRNA). A number of publications addressing the mechanism of RNAi have suggested that, during RNAi, dsRNA breaks into small guide RNAs, and these small RNAs activate and get associated with RNase complex that degrades the target mRNA (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). To determine whether *falcipain* dsRNAs are also processed into small RNAs *in vivo*, the cultured parasites were treated with  $^{32}\text{P}$ -labelled dsRNA from either of the two *falcipains* used in this study. After 24 h of incubation, parasite pellets were processed to get total RNAs and, subsequently, small RNA species were isolated from the total RNAs. The small RNA species were then analysed by polyacrylamide gel electrophoresis. As shown in Fig. 4A, both *falcipain* dsRNAs were processed to  $\approx 25$  nt species. We also observed an additional RNA species of  $\approx 70$  nt in the dsRNA-treated parasites. Such intermediate/longer RNA species have also been observed in other studies, although their role in RNAi has not yet been elucidated (Grishok *et al.*, 2001).

With the development of the *Drosophila in vitro* system, different steps underlying the mechanism of RNAi have been recapitulated *in vitro* (Zamore *et al.*, 2000; Elbashir *et al.*, 2001). In the *in vitro* system, it has been demonstrated that preincubation of the dsRNA in the *Drosophila*



**Fig. 4.** A. Generation of PTGS characteristic  $\approx$  25 nt RNA upon incubation with *falcipain* dsRNAs in parasite culture.  $^{32}\text{P}$ -labelled dsRNA from each *falcipain* was added to *P. falciparum* cultures and incubated for 40 h. As a control, labelled dsRNAs were added to normal RBCs for the same period. Total RNAs were isolated from parasites, and small RNAs were purified from total RNA. The small RNA species were resolved on 15% denaturing acrylamide gel. Lane 1, *falcipain-1* dsRNA-treated parasites; lane 2, *falcipain-2* dsRNA-treated parasites; lane 3, control labelled dsRNA from *falcipain-1*; and lane 4, control labelled dsRNA from *falcipain-2*. B. RNAi *in vitro*. Denaturing agarose gel analysis of radiolabelled *falcipain-2* mRNA incubated for the times indicated with *falcipain-2* dsRNA-treated (i) and untreated parasite lysate (ii).

lysate results in potentiation of nuclease activity for target mRNA degradation (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). To investigate whether similar specific activity was generated in *P. falciparum*, cellular extracts from *falcipain-2* dsRNA-treated and untreated parasites were prepared and incubated with  $^{32}\text{P}$ -labelled *falcipain-2* mRNA in the presence of ATP. Samples from these reaction mixtures were taken at different time points and analysed by agarose gel. As shown in Fig. 4B, extracts from dsRNA-treated parasites efficiently degraded *falcipain-2* transcript, whereas extracts from untreated parasites did not degrade mRNA at all. Indeed, extracts from treated samples degraded mRNA very quickly, i.e. within 30 min of incubation, most of the mRNA was degraded, whereas

extract from untreated parasites did not show any mRNA degradation activity up to 3 h.

## Discussion

The discovery of RNAi is one of the most exciting advances in molecular genetics in recent years (Fire, 1999; Sharp, 2001). In the present study, we attempted RNAi in *P. falciparum*, an intracellular parasite using dsRNAs from the two parasite-specific cysteine protease genes (*falcipain-1* and *falcipain-2*). We chose these genes because the effects of different cysteine protease inhibitors on the morphology and biochemistry of malaria parasites, i.e. enlargement of the food vacuole with large amounts of haemozoin in it and block in haemoglobin degradation, have been well documented (Rosenthal, 1995; Rosenthal *et al.*, 1996; Sijwali *et al.*, 2001). Recombinant Falcipain proteins have also been shown to degrade haemoglobin *in vitro* (Sijwali *et al.*, 2001). More recently, the role of cysteine proteases in the release of malaria parasites from host erythrocytes has also been postulated (Salmon *et al.*, 2001). In the present investigation, the addition of each of the two *falcipain* dsRNAs to *P. falciparum* culture resulted in inhibition of parasite growth, development and enlargement of food vacuoles. These effects were more pronounced when two *falcipain* dsRNAs were put together. Moreover, each of the *falcipain* dsRNAs produced a significant block in the degradation of haemoglobin in the parasite. A non-specific dsRNA from an aminopeptidase-N gene of insect origin did not produce any of these effects. The additive effects produced by two *falcipain* dsRNAs suggested that haemoglobin hydrolysis in parasites is probably a co-operative process involving a number of different enzymes together. The inhibitory effects produced by dsRNA treatment of parasites coincided with a marked reduction in the levels of endogenous mRNA homologous to the dsRNA in these parasites, which is one of the hallmarks of RNAi. To confirm further that these effects resulted from *falcipain* dsRNAs, we simultaneously studied the uptake of labelled *falcipain* dsRNAs by the malaria parasites. Indeed, we observed the accumulation of radioactivity in the saponin-lysed parasite pellets, suggesting uptake of dsRNA by the parasites. Although we observed low levels of uptake of radioactive *falcipain* dsRNAs compared with total input counts, we believe that it is sufficient to produce RNAi. DsRNA molecules do not seem to act stoichiometrically to silence gene expression. In *C. elegans*, it has been shown that two molecules of dsRNA per cell were able to silence an abundantly expressed gene effectively (Fire *et al.*, 1998; Cogoni and Maino, 2000). We also compared the effects produced by *falcipain* dsRNAs on malaria parasites with the effects of E-64, a well-known cysteine

protease inhibitor. Our study showed that various morphological as well as biochemical effects seen on malaria parasites after treatment with *falcipain* dsRNAs were similar to those shown by E-64. These results indicate that RNAi can be an important tool in investigating metabolic events in the parasite life cycle and add *P. falciparum* to the list of organisms in which RNAi has been shown to work successfully. Although substantial inhibitory effects were observed with the highest concentrations of *falcipains* in the present study, we could never achieve 100% efficacy. This could either be attributed to technical limits of the RNAi application or suggest the existence of hidden cysteine proteases in addition to the two taken up in the present study.

The block in haemoglobin degradation observed by silencing the activity of the *falcipain* genes in the present study suggested that both the *falcipain* proteins are required for initial cleavage of haemoglobin. Several different enzymes present in the food vacuole of *P. falciparum* appear to be involved in haemoglobin degradation, but their precise role and hierarchy in the proteolysis of haemoglobin are not clearly understood. It has been suggested that haemoglobin degradation is an ordered process with aspartic proteases leading the proteolytic process, followed by cysteine and other proteases (Goldberg *et al.*, 1990). However, this model has been a matter of debate, and results contrary to the above hypothesis have been reported (Rosenthal, 1995; Shenai *et al.*, 2000). Taken together, the results of the present study also suggest that cysteine proteases might be involved in the initial cleavage of haemoglobin and that their inhibition causes its accumulation in the parasite. In this regard, our results support the work of Rosenthal (1995), who showed that cysteine proteases are involved right from the beginning in the proteolysis of haemoglobin. It is quite possible that both classes of proteases are playing a dominant role initially. However, regardless of whether cysteine, aspartic or both classes of proteases cleave haemoglobin initially inside the food vacuole, the haemoglobin hydrolysis pathways remain an attractive target for the quest for new antimalarials.

Recently, on the basis of studies related to the RNAi mechanism, a two-step RNA degradation process has been proposed. The first step involves a dsRNA endonuclease [ribonuclease III (RNase III)]-like activity that processes dsRNA into RNAs 21–25 nt long called siRNA. In a second step, siRNAs produced in the first step serve as a guide for a different ribonuclease complex, RISC (RNA-induced silencing complex), which cleaves the homologous single-stranded mRNA (Metzke *et al.*, 2001). We wondered whether the mechanism of RNAi is similar in *P. falciparum*. Analysis of small RNA species in the *P. falciparum* culture treated with labelled *falcipain* dsRNAs

showed the generation of  $\approx 25$  nt labelled RNA species. We also showed that parasite extracts prepared from the *P. falciparum* culture treated with *falcipain-2* dsRNA have the ability to cleave *falcipain-2* mRNA, whereas the untreated parasite did not show this nuclease activity. These results thus supported the two-step mechanism of RNAi and also suggested a conservation of both mechanisms and the components of dsRNA-induced gene silencing in a diverse group of organisms. Recent genetic, molecular and mutational studies have provided further support for this hypothesis. Two sets of genes coding for proteins implicated in RNAi have been identified in different organisms. The first set of proteins is the one with homology to tomato RNA-directed RNA polymerase (RdRP): *Neurospora* QDE-1, *C. elegans* EGO-1 and *Araabidopsis* SGS-2/SDE-1. The second set of proteins involved in RNAi includes *Arabidopsis* AGO-1, *Neurospora* QDE-2 and *C. elegans* RDE-1 (Fagard *et al.*, 2000; Elbashir *et al.*, 2001; Grishok *et al.*, 2001). A BLAST search at the PlasmoDB.org site was carried out using tomato RdRP and *Arabidopsis* AGO-1 sequences. A gene related to AGO-1 was identified on chromosome 10 (19186) of *P. falciparum*, which showed 31% identity to putative translation factor EIF-2B. We also found putative RNase III and RdRP sequences in the *P. falciparum* genome, using the *Plasmodium* genome BLASTX text query tool at the PlasmoDB.org site, which showed some homology (29% and 24% respectively) to that of *Dictyostelium discoideum*. The significance of these findings remains to be investigated.

The use of RNAi in *P. falciparum* to dissect gene functions has several advantages over methods requiring the introduction of DNA into cells. Transfection experiments require knowledge of the full sequence of the gene and its flanking regions, whereas for RNAi, dsRNA corresponding to any gene fragments is sufficient to confer the interference effect (Ullu and Tschudi, 2000). Moreover, use of RNAi in *P. falciparum* as well as in other organisms is technically simple and quick, and the result of protein silencing can be obtained within 2–3 days. This contrasts sharply with the time necessary to produce selective gene 'knock-outs' by transfection technology. RNAi in *P. falciparum* also provides a tool for studying the functions of a number of genes together, as shown in the present study. With the availability of a large amount of sequence information for *P. falciparum*, RNAi-based functional genomics can be a useful technique for investigating the biological functions of novel genes. Finally, given the gene-specific feature of RNAi and the ease of inducing RNAi, this methodology may also play an important role in the development of therapeutic applications against malaria. In conclusion, the present study demonstrates that the RNAi technique can be used conveniently for studying

gene function in *P. falciparum*, and RNAi is mechanistically conserved in *P. falciparum* and other organisms.

## Experimental procedures

### RNA preparation

Individual DNA fragments coding for both *falcipain-1* (1.7kb) and *falcipain-2* (1.4kb) genes (GenBank accession numbers AF239801 and M81341 respectively) (Fig. 1A) were amplified by polymerase chain reaction (PCR) using *P. falciparum* genomic DNA as a template and oligonucleotide primer pairs Pf1(1) 5'-ATGGTTGCCATAAAAAGAAATG-3' and Pf1(2) 5'-TTACAAGATAGGATAGAAGAC-3'; and Pf2(1) 5'-ATGGATTACAACATGGATTAT-3' and Pf2(2) 5'-TTATTCAATTAATGGAATGAA-3'. The PCR products were gel purified and cloned into pGEM-T vector (Promega) to generate pGFal-1 and pGFal-2 clones. The clones were sequenced by dideoxy sequencing reactions and used as a template to amplify individual *falcipain* genes using universal and reverse primers (Promega). The PCR products were purified using a PCR purification kit (Qiagen). The purified PCR products were used as a template to generate sense RNA (sRNA) and antisense RNA (asRNA) using T7 and SP6 RNA polymerases (Ambion). To make dsRNAs, equal amounts of ssRNAs and asRNAs were mixed, heated to 65°C and annealed by slow cooling over several hours. Individual RNAs and their dsRNAs were analysed on 1% agarose gel. A 0.9 kb dsRNA from the unrelated aminopeptidase-N gene of insect *Spodoptera litura* was prepared in a similar way to use as a control. To make <sup>32</sup>P-labelled dsRNAs, either sense or antisense strands of *falcipain* genes were internally labelled using 0.3 μM [ $\alpha$ -<sup>32</sup>P]-UTP (3000 Ci mmol<sup>-1</sup>) and mixed with unlabelled complementary strands. [<sup>32</sup>P]-dsRNAs were treated with RNase T<sub>1</sub> to eliminate ssRNA contaminants.

### Parasite culture and RNAi

*Plasmodium falciparum* (strain 3D7) were cultured with human erythrocytes with 5% haematocrit in RPMI media (Gibco) supplemented with 10% human serum using a protocol described previously (Trager and Jensen., 1976). Parasite cultures were synchronized using sorbitol (Lambros and Vanderber, 1979). For analysis of the effects of dsRNAs or L-transepoxy-succinyl-leucylamido-(4-guanidino)-butane (E-64), the synchronized cultures were adjusted to 5% haematocrit with 1% infected red blood cells (RBCs), and 1 ml of these cultures was treated in 24-well culture plates in triplicate in serum-free medium for 30 min with intermittent mixing. Subsequently, human serum was added to these cultures to a final concentration of 10%, and parasites were maintained further for 24 h or 48 h. For the microscopic analysis, smears were made from each well, stained with Giemsa and examined for abnormalities as well as for the number of ring-stage parasites. The number of ring-stage parasites per 1500 RBCs was determined for each well. Parasites with enlarged food vacuoles and haemozoin accumulation were scored as abnormal. To assess the effects of various dsRNAs/inhibitors on parasite metabolic activity, a [<sup>3</sup>H]-

hypoxanthine uptake assay was performed as described previously (Rosenthal *et al.*, 1996). Briefly, 100 μl of parasite cultures (0.8–1.0% parasitaemia) at late ring stage were incubated with dsRNAs in triplicate for 24 h, [<sup>3</sup>H]-hypoxanthine (Dupont-NEN) was added (1.2 μCi per well), and cultures were maintained for an additional 24 h. Cultures of mature parasites were frozen and thawed to lyse the infected RBCs. Lysed cultures were harvested on glass-fibre filters, which were subsequently washed with water, dried with ethanol and counted on a scintillation counter. Uptake of [<sup>3</sup>H]-hypoxanthine by dsRNA-treated parasites was compared with that of control cultures treated with diethyl pyrocarbonate (DEPC)-treated water.

### Analysis of endogenous *falcipain* RNA transcripts

Northern blot analysis was carried out to determine the levels of endogenous *falcipain* transcripts after *falcipain* dsRNA treatment for 48 h. Total RNAs were prepared from treated and untreated malaria parasites using the Qiagen RNA isolation kit. Total RNA (1 μg) was fractionated on 1.2% agarose gel, transferred to a nylon membrane (Hybond N<sup>+</sup>) and hybridized with random prime-labelled *falcipain-1* DNA probe according to a previously described protocol (Kyes *et al.*, 2000). The blot was exposed to X-ray film (Kodak). After exposure, the membrane was deprobed, hybridized with random prime-labelled *falcipain-2* DNA probe and re-exposed to X-ray film.

### Analysis of globin hydrolysis in *P. falciparum* parasites

To determine the effects of *falcipain* dsRNAs or E-64 on haemoglobin degradation in the malaria parasite, *P. falciparum* cultures were treated with these inhibitors for 24 h. After the treatment, parasite-infected erythrocytes were collected by centrifugation and lysed with 0.1% saponin in PBS at 37°C for 15 min. The lysed samples were centrifuged, and the parasite pellets were washed three times with ice-cold PBS to remove erythrocyte cytoplasmic contents. The washed parasite pellets were solubilized in SDS sample buffer containing β-mercaptoethanol, boiled and separated on 15% SDS-PAGE. The gels were stained with Coomassie blue.

### Analysis of *falcipain* dsRNA processing in *P. falciparum*

To determine whether *falcipain* dsRNAs inside *P. falciparum* parasites are processed to small RNAs, as shown for RNAi in the case of other organisms (Zamore *et al.*, 2000), parasite cultures were treated with <sup>32</sup>P-labelled dsRNAs of *falcipain-1* and *-2* separately as described earlier. Treated parasitized erythrocytes were pelleted by centrifugation. Total RNAs were extracted as described earlier. Low-molecular-weight RNAs were extracted from total RNA by precipitation with 5% polyethylene glycol–0.5 M NaCl (Hamilton and Baulcombe, 1999). Low-molecular-weight RNAs were subsequently analysed by electrophoresis through 15% polyacrylamide–7 M urea–0.5% Tris-borate gel. The gel was dried under vacuum and exposed to X-ray film (Kodak).



*Plasmodium falciparum* extract preparations and degradation of mRNA in vitro

DsRNA-mediated gene silencing occurs as a result of activation of RNA-dependent nucleases inside the cells by dsRNA. To show that similar activation is also responsible for RNAi in *P. falciparum*, extracts were prepared from *falcipain-2* dsRNA-treated as well as DEPC water-treated parasites. For the preparation of extracts, intact parasites were collected by saponin lysis and washed three times in ice-cold PBS to remove erythrocyte cytoplasmic components. The washed parasite pellets were lysed by sonication in 200 µl of lysis buffer [100 mM phosphate acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate and 5 mM dithiothreitol (DTT), pH 7.4]. The lysate were centrifuged for 30 min at 15 000 g at 4°C, and the supernatants were flash frozen in liquid nitrogen and stored at -80°C. The supernatants were used for an *in vitro* assay. Typically, the reaction mixtures (50 µl) contained 25 µl of parasite extract and 50 000 c.p.m. of synthetic mRNA as a substrate in a buffer containing 20 mM HEPES, pH 7.3, 110 mM KOAc, 1 mM Mg (OAc)<sub>2</sub>, 3 mM EGTA, 2 mM CaCl<sub>2</sub>, 1 mM DTT and 1 mM ATP. The reaction mixtures were incubated for different time periods at 37°C. Reactions were quenched by the addition of 10 volumes of 2% PK buffer [200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl and 2% (w/v) SDS]. Proteinase K was added to a final concentration of 465 µg ml<sup>-1</sup>. The reactions were extracted with phenol-chloroform-isoamyl alcohol and precipitated with an equal volume of isopropanol. Reactions were analysed by electrophoresis in a formaldehyde-agarose 0.8% gel. mRNA degradation was detected by exposing the agarose gel dried under vacuum to X-ray film (Kodak).

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