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Invited review

Cysteine proteases of malaria parasites

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Abstract

A number of cysteine proteases of malaria parasites have been described, and many more putative cysteine proteases are suggested by analysis of the *Plasmodium falciparum* genome sequence. Studies with protease inhibitors have suggested roles for cysteine proteases in hemoglobin hydrolysis, erythrocyte rupture, and erythrocyte invasion by erythrocytic malaria parasites. The best characterised *Plasmodium* cysteine proteases are the falcipains, a family of papain-family (clan CA) enzymes. Falcipain-2 and falcipain-3 are hemoglobinases that appear to hydrolyse host erythrocyte hemoglobin in the parasite food vacuole. This function was recently confirmed for falcipain-2, with the demonstration that disruption of the falcipain-2 gene led to a transient block in hemoglobin hydrolysis. A role for falcipain-1 in erythrocyte invasion was recently suggested, but disruption of the falcipain-1 gene did not alter parasite development. Other papain-family proteases predicted by the genome sequence include dipeptidyl peptidases, a calpain homolog, and serine-repeat antigens. The serine-repeat antigens have cysteine protease motifs, but in some the active site Cys is replaced by a Ser. One of these proteins, SERA-5, was recently shown to have serine protease activity. As SERA-5 and some other serine-repeat antigens localise to the parasitophorous vacuole in mature parasites, they may play a role in erythrocyte rupture. The *P. falciparum* genome sequence also predicts more distantly related (clan CD and CE) cysteine proteases, but biochemical characterisation of these proteins has not been done. New drugs for malaria are greatly needed, and cysteine proteases may provide useful new drug targets. Cysteine protease inhibitors have demonstrated potent antimalarial effects, and the optimisation and testing of falcipain inhibitor antimalarials is underway.

Keywords: Plasmodium; Malaria; Protease; Proteinase; Peptidase; Cysteine

1. Introduction

Malaria, particularly disease caused by *Plasmodium falciparum*, is one of the most important infections of humans, and its control is currently challenged by increasing resistance to available drugs. New antimalarial drugs, ideally directed against new targets, are needed. Among potential new targets for chemotherapy are *Plasmodium* proteases. Many proteolytic enzymes appear to play key roles in the life cycles of malaria parasites. This review will focus on one class of *Plasmodium* proteases, the cysteine proteases. Recent advances, including the sequencing of *Plasmodium* genomes (http://plasmodb.org) and the development of techniques for manipulating *Plasmodium* genes (Crabb, 2002), have improved our understanding of the cysteine protease repertoire of malaria parasites, and have begun to illuminate specific functions of these enzymes. In parallel with biochemistry advances, drug discovery projects designed to identify new antimalarial cysteine protease inhibitors are underway. This review will survey available information on *Plasmodium* cysteine proteases and prospects for exploitation of this class of enzymes as drug targets. A more detailed review on this topic was also published recently (Rosenthal et al., 2002).

2. Cysteine protease nomenclature

Cysteine proteases are so-named due to the function of a catalytic cysteine, which mediates protein hydrolysis via nucleophilic attack on the carbonyl carbon of a susceptible peptide bond. Cysteine proteases are sub-divided into clans (Barrett and Rawlings, 2001; Barrett et al., 1998).

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Different clans do not share sequence or structural identity and probably arose independently, but they share the use of a cysteine to catalyse the hydrolysis of peptide bonds. Clans are sub-divided into families based on sequence identities and similarities. Clan CA proteases utilise catalytic Cys, His, and Asn residues that are invariably in this order in the primary sequence of the protease. Clan CA, Family C1 (papain-family) cysteine proteases are well characterised for many eukaryotic organisms (Turk et al., 2001), and these are the best characterised cysteine proteases of *Plasmodium*. In *P. falciparum*, analysis of the genome sequence suggests that clan CA cysteine proteases include as many as four falcipains, three dipeptidyl peptidases, nine proteins related to the serine-rich antigen (SERA), and a calpain homolog (Wu et al., 2003) (Table 1). A second clan of interest in *Plasmodium* is clan CD, which utilises a catalytic His-Cys dyad (in this order in the primary sequence) for activity. Clan CD proteases includes caspases in higher organisms, and sequence analyses suggest that members of the C13 and C14 families are present in plasmodia. Clan CE, which is characterised by catalytic residues in the order His, Glu (or Asp), Cys, is also represented in the *P. falciparum* genome.

3. Functions of *Plasmodium* cysteine proteases determined from inhibitor studies

Studies with protease inhibitors have provided valuable information regarding the functions of cysteine proteases in malaria parasites (Rosenthal, 2001). A number of compounds that specifically inhibit cysteine proteases are available. In most cases these inhibitors are directed toward clan CA proteases, but do not offer marked specificity within this large clan. Therefore, they have been most useful to identify cysteine protease functions, rather than the roles of specific enzymes.

The timing of effects of cysteine protease inhibitors provides clues regarding protease functions. In a detailed characterisation of effects of leupeptin and E-64 against cultured *P. falciparum* parasites, both inhibitors were maximally active against mature trophozoites and schizonts, although 8 h incubations during any portion of the life cycle elicited some inhibition of parasite development (Shenai et al., 2002). These results support a critical role for cysteine proteases in trophozoite hemoglobin hydrolysis (see below), but also suggest that additional cysteine protease functions may be important and also targets for inhibitor effects.

3.1. Hemoglobin hydrolysis

The best characterised function of *Plasmodium* cysteine proteases is the hydrolysis of hemoglobin. Erythrocytic malaria parasites multiply asexually, reaching large numbers in the circulation, and causing the clinical manifestations of malaria. During this cycle, parasites take up erythrocyte cytosol through a specialised organelle, the cytostome, transport the cytosol to an acidic food vacuole, and degrade hemoglobin (Francis et al., 1997b). As hemoglobin is processed, its heme component is converted into hemozoin pigment, and globin is hydrolysed to its constituent amino acids. Hemoglobin hydrolysis is necessary to provide amino acids for parasite protein synthesis (Francis et al., 1997b; McKerrow et al., 1993), to maintain the osmotic stability of malaria parasites (Lew et al., 2003), to provide space for the growing intraerythrocytic parasite, and/or for as yet unexplained reasons. Hemoglobin hydrolysis appears to be the result of a cooperative process involving proteases of multiple catalytic classes, including cysteine, aspartic, and metallo proteases (Francis et al., 1997b).

Evidence for a role of cysteine proteases in hemoglobin hydrolysis came from studies of the effects of cysteine protease inhibitors on cultured parasites. Incubating parasites with broadly active cysteine protease inhibitors, such as leupeptin and E-64, caused the food vacuole to swell and fill with dark-staining material (Bailly et al., 1992; Dluzewski et al., 1986; Rosenthal et al., 1988). It appeared that these parasites transported erythrocyte cytosol to the food vacuole normally, but that the processing of hemoglobin was blocked. Indeed, isolation of proteins from parasites treated with cysteine protease inhibitors demonstrated the accumulation of large quantities of undegraded hemoglobin (Rosenthal et al., 1988). With the understanding that multiple classes of proteases participate in hemoglobin hydrolysis, some debate regarding the specific roles of different enyzmes in this process has ensued. Based on some in vitro studies, it has been argued that only aspartic proteases are capable of initial cleavages of intact hemoglobin (Gluzman et al., 1994). However, only cysteine protease inhibitors have been shown to cause the food vacuole swelling that is indicative of a block in hemoglobin processing (Rosenthal, 1995). In addition, analysis of proteins from cysteine protease inhibitor-treated parasites has identified large quantities of intact native hemoglobin in these parasites, suggesting that cysteine proteases participate in initial cleavages of hemoglobin (Gamboa de Dominguez and Rosenthal, 1996).

Cysteine proteases may also contribute indirectly to hemoglobin hydrolysis via the processing of aspartic proteases. The processing of the aspartic proteases plasmepsin I and plasmepsin II was inhibited by tripeptide aldehyde cysteine protease inhibitors, but not by other cysteine protease inhibitors, including leupeptin and E-64 (Francis et al., 1997a). This differential inhibition suggested that a cysteine protease other than falcipain-2 or falcipain-3 mediates the processing of plasmepsins. An alternative explanation is that differential localisation of the inhibitors might explain different intracellular effects. In any event, the protease responsible for plasmepsin processing has not been identified.

3.2. Erythrocyte rupture

Cysteine protease inhibitors have also been shown to inhibit the rupture of erythrocytes that accompanies the completion of the erythrocytic cycle. Cysteine protease activity therefore appears to be required for the release of merozoites, which rapidly invade other erythrocytes to reinitiate the asexual cycle. Older studies showed the accumulation of mature schizonts in cultures treated with leupeptin (Debrabant and Delplace, 1989; Hadley et al., 1983; Lyon et al., 1986). Two recent studies have further explored this area. A careful study of the action of E-64 showed that, in mature schizont-stage parasites, this inhibitor blocked the lysis of the parasitophorous vacuole membrane, which surrounds the intraerythrocytic parasite (Salmon et al., 2001). This result suggested that cysteine protease activity is required for the hydrolysis of parasitophorous vacuole membrane-associated proteins to mediate merozoite release. A second report used different techniques to show that two other cysteine protease inhibitors, leupeptin and antipain, blocked lysis of the erythrocyte membrane (Wickham et al., 2003). Considering these results together, it appears that the release of merozoites is a two-step process, requiring hydrolysis of proteins associated with the parasitophorous vacuole and erythrocyte membranes, although the sequence of these two steps is in dispute. Cysteine protease inhibitors with broad specificities inhibited lysis of parasitophorous vacuole (E-64) and erythrocyte (leupeptin, antipain) membranes. Differential effects of these inhibitors may have been due to differences in inhibitor specificities, suggesting that different proteases act upon targets in the two membranes. In particular, leupeptin and antipain, but not E-64, inhibit some serine proteases. Alternatively, differences in access of inhibitors to different intracellular targets may explain differences in effects. In any event, these results suggest that cysteine proteases act against proteins associated with the parasitophorous vacuole and erythrocyte membranes to facilitate erythrocyte rupture at the completion of the intraerythrocytic cycle.

3.3. Erythrocyte invasion

Most reports have not identified an effect of cysteine protease inhibitors on the invasion of erythrocytes by merozoites (Rosenthal, 2001). Rather, this process has generally been shown to be inhibited by serine protease inhibitors such as chymostatin (Rosenthal, 2001). Older literature is confused by some reports that did not clearly distinguish between inhibitor effects on erythrocyte invasion and on erythrocyte rupture. Studies in this area are complicated by the fact that merozoites that retain invasive properties cannot readily be obtained from *P. falciparum*. However, with *Plasmodium knowlesi* and *Plasmodium chabaudi*, species from which invasive merozoites can be obtained, the serine protease inhibitor chymostatin, but not the cysteine protease inhibitors leupeptin and E-64, inhibited erythrocyte invasion (Breton et al., 1992; Hadley et al., 1983). A new report has added a new perspective in this area. Using an inhibitor library and evaluations of competitive binding to measure interaction with falcipain-1, a specific inhibitor of falcipain-1 was identified, and this inhibitor was shown to block invasion of erythrocytes by merozoites (Greenbaum et al., 2002). However, interpretation of these results has been complicated by consideration of additional studies of falcipain-1 and this specific inhibitor, as will be discussed below. Thus, it remains unclear whether cysteine proteases are required for erythrocyte invasion.

3.4. Non-erythrocytic parasite stages

Few data are available on cysteine protease functions in non-erythrocytic parasite stages. During gametogenesis, hydrolysis of the gametocyte surface protein Pfs230 generates two polypeptides which remain associated with the newly formed gamete (Williamson et al., 1996). The cleavage of Pfs230 is blocked by the membrane-permeant cysteine protease inhibitor E-64d, suggesting that a cysteine protease is responsible for this cleavage, but the relevant enzymes have not been identified (Brooks and Williamson, 2000). Recently, the knockout of falcipain-1 was shown to lead to markedly decreased oocyst production, suggesting a specific role for this protease in sexual-stage parasites (Eksi et al., 2004).

4. Falcipain cysteine proteases

The best characterised *Plasmodium* cysteine proteases are the falcipains, a family of four papain-family (clan CA, family C1) enzymes of *P. falciparum*. The genome sequence shows that there are four falcipains, falcipain-1, which is encoded on chromosome 14, and three other proteases whose genes are located within a 12 kb stretch of chromosome 11 (Table 1). These proteases are named falcipain-2, falcipain-2′ (which has 99% homology with falcipain-2 in the catalytic domain), and falcipain-3. The falcipains are all fairly typical papain-family cysteine proteases, but they have some unusual features for this family, including unusually large prodomains, predicted membrane spanning sequences within the prodomains, and an unusual insertion between highly conserved residues near the carboxy terminus (Rosenthal and Nelson, 1992; Shenai et al., 2000; Sijwali et al., 2001b) (Fig. 1A). The falcipains differ most notably in that homology between falcipain-1 and the other falcipains is relatively low (~40% identity between the catalytic domains). Falcipain-2 and falcipain-3 are much more similar in sequence (68% identity), share similarly sized prodomains (that of falcipain-1 is much longer), and include an unusual amino-terminal extension of the catalytic domain that is not found in falcipain-1 (see below). Based on these

Table 1
Summary of predicted *Plasmodium falciparum* cysteine protease genes and characterised cysteine proteases

Clan	Family	Protease/homolog	Ch.[a]	Gene ID	Purif.[b]		Activity[c]	KO phenotype[d]	Putative function (eryth. parasites)	Stage of transcription[e]		Stage of protein expression[f]	
					Nat.	Rec.				Eryth.	Other	Immunobl.	Proteomic screen
CA	C1	Falcipain-1	14	PF14_0553		+	CP	Nl development	Not essential	R	M/Sp/G	R/T/S	Sp
		Falcipain-2	11	PF11_0165	+	+	CP	Transient block Hb hydrolysis	Hb hydrolysis	T	M/G/Sp	T	T
		Falcipain-2′	11	PF11_0161					Hb hydrolysis	R/T	M		T
		Falcipain-3	11	PF11_0162		+	CP	Lethal?	Hb hydrolysis	T/S	M/Sp/G	T/S	T/G
		Dipeptidyl pep.	4	PFD0230c						S	M/Sp		M
		Dipeptidyl pep.	11	PF11_0174	+		CP	Lethal?	Hb hydrolysis	R/T	Sp/M/G		T/M
		Dipeptidyl pep.	12	PFL2290w						S	G		T/G
		SERA-1	2	PFB0325c						R	Sp/M/G		Sp
		SERA-2	2	PFB0330c				Nl development	Not essential	T/S	M/Sp/G		M/T
		SERA-3	2	PFB0335c				Nl development	Not essential	T/S	M	T/S	None
		SERA-4	2	PFB0340c				Lethal?	Eryth. rupture?	S	M/Sp/G	T/S	M/T
		SERA-5	2	PFB0345c		+	SP	Lethal?	Eryth. rupture?	S	M	T/S	Sp
		SERA-6	2	PFB0350c				Lethal?	Eryth. rupture?	S	M/Sp/G	T/S	G/T
		SERA-7	2	PFB0355c				Nl development	Not essential	S	Sp/M/G		None
		SERA-8	2	PFB0360c				Nl development	Not essential	S	Sp/G		None
		SERA-9	9	PFI0135c						T/S	Sp/M/G		Sp
	C2	Calpain	13	MAL13P1.310						R	M/Sp/G		None
	C12	UCH1	11	PF11_0177						T/S	G/M/Sp		Sp
		UCH1	14	PF14_0577						T/S	Sp/M/G		T
	C19	UCH2	1	PFA0220w						S/R	M/Sp/G		Sp
		UCH2	4	PFD0165w						R/T/S	Sp/M/G		Sp
		UCH2	4	PFD0680c						T/S	G/M		M
		UCH2	5	PFE1355c						T/S	M/G/Sp		M/T/G
		UCH2	5	PFE0835w						S/R	M		M
		UCH2	7	MAL7P1.147						T	Sp/G/M		G/M/Sp/T
		UCH2	9	PFI0225w						R/T/S	Sp/M/G		Sp
		UCH2	13	PF13_0096						R/T/S	Sp/M		None
		UCH2	14	PF14_0145						R	M/Sp/G		Sp
CD	C13	GPI:Pr Tr	11	PF11_0298						S	G		G
	C14	Metacaspase	13	PF13_0289						R	Sp/G		None
		Metacaspase	14	PF14_0363						S	G/Sp/M		G
CE	C48	Sumo 1	12	PFL1635w						R/T/S	M/Sp/G		G/M
		Ulp 2	8	MAL8P1.157						T	G/M/Sp		Sp

Data shown are based on information from multiple sources, including primary research papers referenced in this review, genomic (Bozdech et al., 2003; Le Roch et al., 2003) and proteomic (Florens et al., 2002) screens of *Plasmodium falciparum*, and summaries on the PlasmoDB web site (http://plasmodb.org). Blanks indicate that data are unavailable or inconclusive. For genomic and proteomic screens, the parasite stages are listed based on the relative quantities of signals identified, but it is cautioned that the biological relevance of these results are uncertain. Abbreviations: KO, knockout; Eryth., erythrocyte or erythrocytic; Immunobl., immunoblots; Nat., native; Rec., recombinant; R, ring; T, trophozoite; S, schizont; M, merozoite; G, gametocyte; Sp, sporozoite; CP, cysteine protease; SP, serine protease; Nl, normal.

[a] *Plasmodium falciparum* chromosome number.

[b] As indicated, active protein has been purified from native (Nat.) or recombinant (Rec.) material.

[c] General properties of proteases that have been purified and characterised.

[d] For characterisations of KO phenotypes, 'Lethal?' indicates that surviving transfectants could not be obtained despite repeated attempts with protocols that successfully generated transfectants with other gene disruptions.

[e] Information is summarised based on two large genomics screens (Bozdech et al., 2003; Le Roch et al., 2003).

[f] Data are based on published immunoblot results and a proteomic screen (Florens et al., 2002).

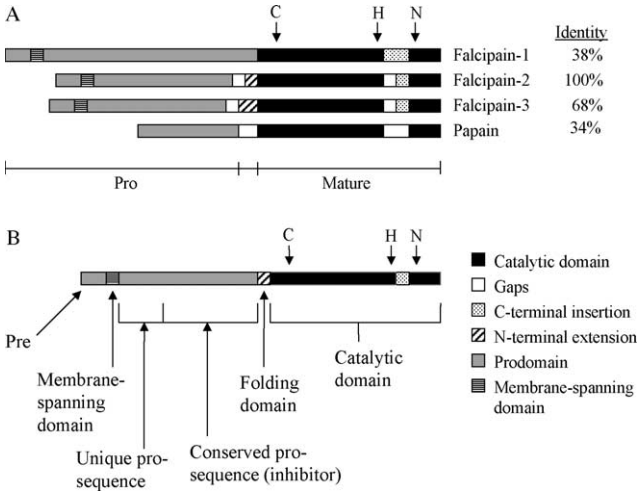

Fig. 1. (A) Schematic comparison of falcipains and papain. Domains are labeled in the key. Gaps to allow alignment are in white. Catalytic amino acids are indicated with arrows and the one-letter code. Amino acid identities between falcipain-2 and other catalytic domains are shown. (B) Domains of falcipain-2. Domains and predicted functions are indicated.

comparisons and similar findings with homologs in other *Plasmodium* species, it seems appropriate to consider two distinct groups of falcipains, the falcipain-1 and falcipain-2/3 sub-families.

4.1. Falcipain homologs in other plasmodial species

Genes encoding falcipain homologs have been identified in numerous other *Plasmodium* species (Rosenthal et al., 2002). In general, it appears that all of the species contain a single homolog of falcipain-1, with varied numbers of falcipain-2/3 homologs. In *Plasmodium vivax*, the second most important human malaria parasite, a single homolog of falcipain-1 (72% identity between the catalytic domains) (Rosenthal et al., 1994) and two homologs of falcipain-2/3 (60–70% identity) (Na et al., 2004) have been characterised. Analysis of murine malaria parasites, including *Plasmodium vinckei*, has identified only single homologs of falcipain-1 (Rosenthal, 1993) and falcipain-2/3 (Singh et al., 2002). Identity between human and rodent parasite proteases is somewhat lower than that between *P. falciparum* and *P. vivax* proteases, but nonetheless identities within sub-families are generally over 50% (in catalytic domains), compared with 30–40% identities between falcipain-1 and falcipain-2/3 sub-families and between any of the falcipains and other papain-family proteases (Rosenthal et al., 2002).

4.2. Expression of falcipains by erythrocytic parasites

Plasmodial proteases have been difficult to purify due to limitations on available starting material and the complexity of separating numerous similar host and parasite enzymes. Fairly recently, falcipain-2 was purified by affinity chromatography (Shenai et al., 2000). This purification made clear the fact that falcipain-2 is responsible for over 90% of the cysteine protease activity that is identified in trophozoite lysates with standard peptidyl substrates (Shenai et al., 2000). Abundant falcipain-3 is also expressed by erythrocytic parasites, but it is relatively inactive against peptidyl substrates (Sijwali et al., 2001b). Falcipain-2 and falcipain-3 differ in their timing of expression, as falcipain-2 is expressed about 12 h earlier than falcipain-3, with maximal expression in early trophozoites for falcipain-2 and late trophozoites for falcipain-3 (Sijwali and Rosenthal, 2004; Sijwali et al., 2001b). Immunoblots have also identified falcipain-1 expression across the erythrocytic cycle (Sijwali and Rosenthal, 2004). This enzyme was localised to merozoites by immunofluorescence microscopy (Greenbaum et al., 2002), and transcription of the gene was maximal in ring forms (Bozdech et al., 2003; Le Roch et al., 2003). However, a proteomic analysis only identified falcipain-1 peptides in sporozoites (Florens et al., 2002).

4.3. Biochemical characterisation

Biochemical characterisation of falcipain-2 and falcipain-3 has been expedited by the development of efficient systems for the heterologous expression of these two proteases in *Escherichia coli*, followed by refolding of the active enzymes (Shenai et al., 2000; Sijwali et al., 2001a,b). The biochemical features of the two enzymes are very similar, but not identical, and differences offer clues to potential differences in functions. Both falcipain-2 and falcipain-3 have acidic pH optima, consistetent with activity in the acidic food vacuole. As with most other papain-family proteases, the P_2 position of substrates is most important for specificity, and both proteases prefer peptidyl substrates with leucine at this position. Falcipain-2 and falcipain-3 differ in that falcipain-2 is much more active against peptidyl substrates, is uniquely able to activate and undergo autohydrolysis at neutral pH, and is more stable at neutral pH. Results with recombinant proteins are consistent with evaluations of parasite lysates, where immunoblotting identifies principally mature falcipain-2, but large amounts of pro-falcipain-3 (Sijwali et al., 2001b). Among subcellular fractions, vacuolar fractions principally contain mature enzymes and membrane fractions principally contain proenzymes of both falcipain-2 and falcipain-3. These results suggest that the proteases are synthesised as integral membrane proteins, and subsequently hydrolysed to release soluble active proteases. Hydrolysis is probably autocatalytic, as seen with the recombinant proteases. Both falcipain-2 and falcipain-3 have been localised to the food vacuole by cell fractionation (Shenai et al., 2000; Sijwali et al., 2001b) and immunofluorescence microscopy (E. Dahl, pers. comm.) methods. For falcipain-3, it appears that hydrolysis is delayed until the protease enters an acidic environment, presumably the food vaucole. For falcipain-2, which undergoes autohydrolysis in vitro at neutral pH, processing appears to occur earlier in its transport pathway,

perhaps allowing some active enzyme to act against cytosolic targets such as erythrocyte cytoskeletal proteins (see below).

4.4. Hydrolysis of natural substrates

Considering biologically relevant activities, both falcipain-2 and falcipain-3 hydrolyse native hemoglobin and denatured globin, with maximal activity near the pH (~5.2) of the food vacuole (Shenai and Rosenthal, 2002). As is typical for cysteine proteases, maximal activity requires a reducing environment, although relatively low concentrations of glutathione that are likely physiological are adequate to support this activity. Falcipain-2 has also been shown to hydrolyse the erythrocyte cytoskeletal proteins band 4.1 and ankyrin at neutral pH, suggesting an additional role for this protease in erythrocyte rupture (Dua et al., 2001; Hanspal et al., 2002). As noted above, the ability of falcipain-2 to undergo autohydrolysis and activation at neutral pH may foster its hydrolysis of erythrocyte cytoskeletal proteins, which presumably occurs in the neutral erythrocyte cytosol. However, a relevant biological role for falcipain-2 in this process has not been confirmed.

4.5. Functions of different falcipain domains

Some information is now available regarding functions of different domains of the falcipains (Fig. 1B). The mature forms of falcipain-2, falcipain-3, and some homologs from other *Plasmodium* species are capable of refolding to active enzymes after expression in *E. coli* and refolding by dilution in alkaline buffer (Sijwali et al., 2001a). These are the only papain-family proteases known to be capable of refolding without their prodomains (Bromme et al., 2004). However, correct folding does require the presence of a small (17 amino acids in falcipain-2) amino-terminal extension (Sijwali and Rosenthal, 2004). Interestingly, this extension can mediate correct folding either when it is included with a recombinant catalytic domain (at its usual location immediately upstream of the catalytic domain) or when it is included in the refolding buffer as a separate folding domain-prodomain polypeptide (Sijwali and Rosenthal, 2004). All falcipain-2/3 proteases, but no other known papain-family proteases (and no falcipain-1 sub-family enzymes) contain an ~20 amino acid extension at the amino-terminus of the catalytic domain. The 14–15 amino acids immediately upstream of the catalytic domain, beginning with a fully conserved Tyr, are required to mediate folding (Pandey et al., 2004). The amino-terminal domains show only moderate homology between members of the falcipain-2/3 sub-family, but there is functional conservation, as chimeras of the falcipain-2 catalytic domain with folding domains from other members of the family folded with similar kinetics (Pandey et al., 2004). The folding domain was not required for activity once folding had occurred, and interaction between catalytic and folding domains were independent of the active site (Pandey et al., 2004). As is the case with other papain-family proteases, the prodomain of falcipain-2 is a potent inhibitor of the enzyme (Sijwali et al., 2002). Since the prodomains of all falcipains are unusually long, with conservation with papain-family proteases only in their downstream portions, it is likely that upstream prodomains have *Plasmodium*-specific functions, such as the mediation of protease trafficking, but no experimental data are yet available in this area.

4.6. Biological roles of falcipains—RNA interference (RNAi) studies

Functions of cysteine proteases have been suggested from inhibitor studies, as discussed above, but it has been difficult to distinguish functions for similar enzymes. Recently, advances have been made in characterising protease functions through genetic approaches. One approach has been RNAi. In a recent report, RNAi directed against either falcipain-1 or falcipain-2 inhibited the development of erythrocytic parasites, with accumulation of undegraded hemoglobin and the appearance of abnormal vacuoles that resembled, but were probably not identical to those caused by incubation with cysteine protease inhibitors (Malhotra et al., 2002). Similar results were seen after treating mice infected with *Plasmodium berghei* with short interfering RNAs encoding the *P. berghei* homologs of falcipain-1 and falcipain-2 (Mohmmed et al., 2003); in this case parasites developed large vacuoles, although murine malaria progressed similarly in treated and control animals. These experiments suggest specific roles for falcipain-1 and/or falcipain-2 in parasite hemoglobin hydrolysis, consistent with models based on biochemical results.

4.7. Biological roles of falcipains—disruption of the falcipain-2 gene

More definitive results have come from recent gene disruption studies. Disruption of the falcipain-2 gene was accomplished by transfecting parasites with a selectable plasmid encoding a truncated falcipain-2 gene and selection of mutant parasites (Sijwali and Rosenthal, 2004). Of note, only the falcipain-2 gene, and not that encoding the nearly identical copy falcipain-2′ was disrupted. Falcipain-2 knockout early trophozoites had markedly diminished cysteine protease activity and swollen, dark-staining food vacuoles, consistent with a block in hemoglobin hydrolysis, as caused by cysteine protease inhibitors. However, more mature knockout parasites had normal morphologies. The knockout parasites had absent expression of falcipain-2, but expression of a cross-reactive protein, falcipain-2′, in more mature parasites. Expression of other falcipains and plasmepsin aspartic proteases was similar in wild type and knockout parasites. Although the multiplication rates of wild type and knockout parasites were similar, the knockout

parasites were about three times more sensitive to the cysteine protease inhibitors E-64 and leupeptin, and, remarkably, over 50-fold more sensitive to the aspartic protease inhibitor pepstatin. These results assign a specific function for falcipain-2, the hydrolysis of hemoglobin in trophozoites. This is the first proven function for a plasmodial protease. In addition, they highlight the cooperative action of cysteine and aspartic proteases in hemoglobin degradation by malaria parasites. It appears that the survival of falcipain-2 knockout parasites was due to increased expression of falcipain-2′ and/or scheduled later expression of falcipain-3. In the case of falcipain-3, which has only a single copy gene, knockouts following similar techniques have to date been unsuccessful, suggesting, but not proving an essential function for this protease.

4.8. Biological roles of falcipains—disruption of the falcipain-1 gene

Disruption of the falcipain-1 gene was recently accomplished by two groups using similar methods to those for falcipain-2 (Sijwali et al., 2004; Eksi et al., 2004). Falcipain-1 knockout parasites developed at the same rate as control parasites, with no obvious morphological alterations. Considering prior reports regarding potential roles for cysteine proteases, and in particular falcipain-1 (Greenbaum et al., 2002), it was of interest to evaluate parasite development and the effects of protease inhibitors in control and knockout parasites across the erythrocytic life cycle. Knockout parasites had rates of schizont rupture, merozoite invasion of erythrocytes, and multiplication that were the same as those of control parasites. Treatment of control and knockout trophozoites with cysteine protease inhibitors led to the development of swollen, dark-staining food vacuoles. Treatment of early schizonts caused a block in erythrocyte rupture, but treatment of early or mature schizonts did not appear to block the subsequent invasion of erythrocytes by merozoites. Importantly, results with standard cysteine protease inhibitors (E-64 and E-64d) and a compound previously shown to be a specific inhibitor of falcipain-1, YA29 (Greenbaum et al., 2002), were the same. Indeed, YA29 was not fully specific, as it inhibited recombinant falcipain-2 at mid-micromolar concentrations. These results indicated that falcipain-1 is not essential for erythrocytic malaria parasites and suggested that cysteine proteases may not play a key role in merozoite invasion of erythrocytes. Falcipain-1 RNA (Sijwali et al., 2001b) and protein (Sijwali et al., 2004) are expressed in erythrocytic parasites. However, the apparent lack of effect of the falcipain-1 knockout on erythrocytic parasites, markedly decreased oocyst production by a falcipain-1 knockout clone (Eksi et al., 2004), and the identification of falcipain-1 peptides in a proteomic screen only in sporozoites (Table 1) suggest that the principal roles of falcipain-1 may be in non-erythrocytic stage parasites.

5. Other clan CA cysteine proteases of malaria parasites

The falcipains are the only proven *Plasmodium* cysteine proteases, but the *P. falciparum* genome sequence suggests that a number of other cysteine proteases are present, as will be discussed below.

5.1. Dipeptidyl peptidases

Dipeptidyl peptidases are a family of exopeptidases that remove dipeptides from the amino-termini of polypeptides (Turk et al., 1998). The prototype for this family is the lysosomal protease cathepsin C. The *P. falciparum* genome includes three sequences with homology with dipeptidyl peptidases (Table 1). Recently, the product of one of these genes was purified and named *P. falciparum* dipeptidyl aminopeptidase I (Klemba et al., 2004). As expected based on related proteases, this enzyme cleaved dipeptide substrates. It was localised to the food vacuole, suggesting that it contributes to late steps in hemoglobin hydrolysis. Overall identity between the three sequences is only about 30%. Identity with human cathepsin C is also about 30%. Identity with the three falcipains is 20–25%. If the *P. falciparum* dipeptidyl peptidase genes encode active enzymes, they probably act as exopeptidases. Their functions could include the hydrolysis of host and parasite proteins. One possibility is that one or more of these proteases contributes to the hydrolysis of hemoglobin, either in the food vacuole or in the cytosol (where the final cleavages of peptides to single amino acids are believed to take place (Kolakovich et al., 1997)).

5.2. Calpain homolog

Calpains of higher organisms are cysteine proteases that are calcium-dependent and contain both catalytic and calcium binding domains (Perrin and Huttenlocher, 2002). Homologs in other organisms may not have calcium dependence. Little is known about biological functions of calpains in any system, although roles in cell motility are theorised (Glading et al., 2002). The *P. falciparum* genome sequence encodes a single calpain homolog, although no biochemical data are available, and it is not clear whether the calpain molecule is expressed or catalytically active in any parasite stage.

5.3. SERAs

The SERAs are a group of similar proteins of unknown function. It was reported some years ago that the serine-rich antigen (SERA, now named SERA-5 (Bzik et al., 1988)), an immunogenic protein and potential vaccine component, had similarities in sequence with cysteine proteases. A second member of the family, serine-rich protein homolog (SERPH or SERA-6 (Knapp et al., 1991)), was described soon thereafter. Subsequent sequencing efforts have identified

an array of eight SERA-family proteins on chromosome 2, and a single additional member of the family on chromosome 9 (Table 1). In all cases, the proteins contain a 'protease domain', equivalent in size and in some sequence features to a papain-family catalytic domain, located within a much larger protein without apparent similarity to cysteine proteases in domains other than the protease domain (i.e. there is no region homologous to the papain prodomain). Within the protease domain, all SERA-family proteins share modest homology with papain-family proteases (and therefore with falcipains), but it is noteworthy that canonical clan CA active site residues are either fully conserved (e.g. in the case of SERA-6) or substituted with specific amino acids (e.g. a replacement of the catalytic Cys with Ser in SERA-5). Homologs of SERAs are also seen in other *Plasmodium* species (Gor et al., 1998; Hodder et al., 2003; Kiefer et al., 1996). A number of the SERAs are expressed in erythrocytic parasites, with maximal expression of SERA-5, but also clear expression of SERA-3, 4, and 6, primarily in the trophozoite and schizont stages (Miller et al., 2002; Aoki et al., 2002). Furthermore, gene disruption studies suggested that SERA-4, 5, and 6 are most likely to be essential, as other SERA genes, but not the genes encoding SERA-4, 5, and 6, could be disrupted in cultured parasites (Miller et al., 2002). Recent studies have explored the potential function of SERA-5 as a protease (Hodder et al., 2003). SERA-5 was heterologously expressed in *E. coli* and refolded, and then studied for proteolytic activity. Interestingly, this protein, which includes a cysteine protease scaffold but replacement of the canonical Cys by Ser, exhibited serine protease (chymotrypsin-like) activity, with autohydrolysis inhibited by serine protease inhibitors and modest activity against peptidyl serine protease substrates (Hodder et al., 2003). SERA-6 contains all canonical cysteine protease amino acids, and so it is likely a cysteine protease, but biochemical evidence to support this suggestion is lacking. Since cysteine protease inhibitors block erythrocyte rupture (see above), and as both SERA-5 and SERA-6 have been localised to the parasitophorous vacuole that surrounds mature schizonts (Delplace et al., 1987; Knapp et al., 1991), these proteins, and perhaps other SERAs, may be responsible for proteolytic cleavages required for this process.

6. Clan CD cysteine proteases

Clan CD proteases of protozoans have recently been reviewed in detail (Mottram et al., 2003). Based on evaluation of the genome sequence, *P. falciparum* may express a number of clan CD proteases, but none have been well characterised or demonstrated to have enzymatic activity (Table 1). Considering roles in other biological systems, clan CD proteases are likely to have tighter substrate specificity than clan CA enzymes, suggesting roles in fine regulation of parasite metabolism (Mottram et al., 2003). This high level of specificity suggests promise for clan CD protease inhibitors as highly specific antimalarial drugs. However, at present our understanding of this group of proteases in *P. falciparum* is based only on sequence comparisons and inferences from other organisms, and much more research is needed to determine the roles of these enzymes and their potential as chemotherapeutic targets.

7. Potential for cysteine protease inhibitors as antimalarial drugs

As evidence indicating that cysteine proteases play essential roles in malaria parasites accumulates, an obvious consideration is the inhibition of these enzymes to treat malaria. A number of older studies have supported this concept, with the demonstration that cysteine protease inhibitors have potent in vitro and in vivo antimalarial effects (Rosenthal, 2001). Specifically, peptidyl fluoromethyl ketone (Rockett et al., 1990; Rosenthal et al., 1991, 1993), vinyl sulfone (Olson et al., 1999; Rosenthal et al., 1996; Shenai et al., 2003), and aldehyde (Lee et al., 2003) inhibitors inhibited falcipain activities and blocked the development of cultured parasites at nanomolar concentrations. Some non-peptide falcipain inhibitors showed more modest antiparasitic activity (Dominguez et al., 1997; Li et al., 1995; Ring et al., 1993). In nearly all cases with potent inhibitors, the inhibition of parasite development was accompanied by a specific block in hemoglobin hydrolysis, marked by the appearance of swollen, dark-staining food vacuoles. More recent analysis has shown that antiparasitic effects correlate with potent inhibition of falcipain-2 and falcipain-3 (Shenai et al., 2003). Many potent inhibitors also exert strong inhibition of homologous enzymes from *P. vivax* (Na et al., 2004) and *P. vinckei* (Rosenthal et al., 1993), although specificities may vary, particularly between proteases of human and murine parasites. Cysteine protease inhibitors have also exhibited antimalarial effects in vivo. Treatment of *P. vinckei*-infected mice with fluoromethyl ketone (Olson et al., 1999; Rosenthal et al., 1993), vinyl sulfone (Olson et al., 1999), and aldehyde (Lee et al., 2003) inhibitors led to partial or complete protection against lethal malaria. Inhibitors of cysteine and aspartic proteases showed synergistic antimalarial effects both in vitro (Bailly et al., 1992; Semenov et al., 1998) and in vivo (Semenov et al., 1998), suggesting the possibility of combined protease inhibitor antimalarial therapy. Extensive studies of the antimalarial activity of non-peptidyl inhibitors of falcipains are now underway. Concerning the potential for resistance to antimalarial cysteine protease inhibitors, a recent study described the selection of parasites resistant to a vinyl sulfone falcipain inhibitor, but the selection was slow, and the mechanism of resistance was complex (Singh and Rosenthal, 2004). This result might indicate that resistance

to antimalarial cysteine protease inhibitors will be slow to develop, but the best means of avoiding resistance will likely be the use of combination antimalarial therapy.

8. Summary and conclusions

Our understanding of the cysteine protease repertoire of malaria parasites has increased markedly in recent years. However, detailed information is only available for the falcipains, a group of papain-family proteases. Recent biochemical and molecular studies indicate that falcipain-2 and probably also falcipain-3 are hemoglobinases, and that these enzymes appear to be appropriate targets for antimalarial chemotherapy. Efforts to optimise falcipain inhibitors as antimalarials are currently underway. The SERA proteins constitute an interesting group of putative serine and/or cysteine proteases, with the timing of expression suggesting roles in erythrocyte rupture by mature parasites. Multiple other putative *Plasmodium* cysteine proteases are potential chemotherapeutic targets, and their characterisation should expedite drug discovery.

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