Expression and characterization of the *Plasmodium falciparum* haemoglobinase falcipain-3

Puran S. SIJWALI, Bhaskar R. SHENAI, Jiri GUT, Ajay SINGH and Philip J. ROSENTHAL[1]

Department of Medicine, San Francisco General Hospital, University of California, San Francisco, Box 0811, San Francisco, CA 94143-0811, U.S.A.

In the malaria parasite *Plasmodium falciparum*, erythrocytic trophozoites hydrolyse haemoglobin to provide amino acids for parasite protein synthesis. Cysteine protease inhibitors block parasite haemoglobin hydrolysis and development, indicating that cysteine proteases are required for these processes. Three papain-family cysteine protease sequences have been identified in the *P. falciparum* genome, but the specific roles of their gene products and other plasmodial proteases in haemoglobin hydrolysis are uncertain. Falcipain-2 was recently identified as a principal trophozoite cysteine protease and potential drug target. The present study characterizes the related *P. falciparum* cysteine protease falcipain-3. As is the case with falcipain-2, falcipain-3 is expressed by trophozoites and appears to be located within the food vacuole, the site of haemoglobin hydrolysis. Both proteases require a reducing environment and acidic pH for optimal activity, and both prefer peptide substrates with leucine at the P_2 position. The proteases differ, however, in that falcipain-3 undergoes efficient processing to an active form only at acidic pH, is more active and stable at acidic pH, and has much lower specific activity against typical papain-family peptide substrates, but has greater activity against native haemoglobin. Thus falcipain-3 is a second *P. falciparum* haemoglobinase that is particularly suited for the hydrolysis of native haemoglobin in the acidic food vacuole. The redundancy of cysteine proteases may offer optimized hydrolysis of both native haemoglobin and globin peptides. Consideration of both proteases will be necessary to evaluate cysteine protease inhibitors as antimalarial drugs.

Key words: cysteine protease, haemoglobin, malaria, papain, trophozoite.

INTRODUCTION

Malaria, in particular that caused by *Plasmodium falciparum*, remains one of the most important infections of humans, and new antimalarial drugs are urgently needed. During the erythrocytic cycle of infection that is responsible for the clinical manifestations of malaria, *P. falciparum* trophozoites hydrolyse host haemoglobin within an acidic food vacuole to provide amino acids for parasite protein synthesis [1,2]. Cysteine protease inhibitors block haemoglobin hydrolysis and parasite development, indicating that parasite cysteine protease activity is required for this essential process [3,4]. Proteases of other mechanistic classes, including aspartic [5] and metallo- [6] proteases, are also present in the food vacuole, and also appear to participate in haemoglobin processing. In *P. falciparum*, three papain-family cysteine protease genes have been identified. Falcipain-1 was identified in erythrocytic parasites and shown to hydrolyse haemoglobin [7,8], but difficulties with heterologous expression and apparent low abundance of the protease in trophozoites have limited its characterization. Two more closely related protease genes, *falcipain-2* and *falcipain-3*, were identified recently [9]. Falcipain-2 was shown to be the principal trophozoite cysteine protease identified in earlier studies and an active haemoglobinase [3,9]. Falcipain-3 has not previously been characterized.

Since cysteine protease inhibitors block parasite development *in vitro* and *in vivo*, the plasmodial cysteine proteases are promising chemotherapeutic targets [3,10]. Rational drug discovery requires well-characterized drug targets, so it was an important priority to determine the biochemical properties and biological role of falcipain-3. In particular, we were interested in defining differences between falcipain-2 and falcipain-3, and thus in explaining the redundant expression of these two similar enzymes by *P. falciparum*. We have found that the enzymes have key biochemical differences and that their joint expression appears to provide optimal hydrolysis of haemoglobin and possibly other cellular targets by erythrocytic parasites.

EXPERIMENTAL

Materials

Benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl coumarin (Z-Phe-Arg-AMC) and Gly-Phe-Gly-semicarbazone were from Bachem. All other peptide substrates were a gift from Dr David Tew (GlaxoSmithKline, King of Prussia, PA, U.S.A.). Z-Phe-Arg-fluoromethyl ketone (Z-Phe-Arg-FMK) was a gift from Dr Robert Smith (Prototek, Dublin, CA, U.S.A.). All other reagents were from Sigma or as mentioned in the text.

Parasite culture and preparation of parasites

W2-strain *P. falciparum* parasites were cultured in RPMI 1640 medium with human erythrocytes and 10 % (v/v) human serum [11]. Synchrony was maintained by serial sorbitol treatments [12]. To obtain free parasites, infected erythrocytes were washed with ice-cold PBS, lysed with 0.1 % saponin in ice-cold PBS for 5 min, centrifuged (12000 ***g*** for 10 min at 4 °C) and washed three times with ice-cold PBS. To obtain parasite extracts, free parasites were suspended in extraction buffer [20 mM Bis-Tris/HCl (pH 6.0), 1 mM EDTA, 0.1 mM PMSF and 10 μM pepstatin], subjected to two freeze/thaw cycles, centrifuged at 12000 ***g*** for 10 min at 4 °C and the supernatant was collected. Subcellular

Abbreviations used: Z, benzyloxycarbonyl; AMC, 7-amino-4-methyl coumarin; FMK, fluoromethylketone; BLAST, basic local alignment search tool; ORF, open reading frame; RT, reverse transcriptase; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol;

[1] To whom correspondence should be addressed (e-mail rosnthl@itsa.ucsf.edu).

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fractions were obtained at 0 °C by modifications of previously described methods [13,14] using buffers supplemented with Complete Protease Inhibitor Cocktail tablets (Roche). A trophozoite pellet was washed three times with PBS and divided into two equal parts. One half was resuspended in SDS/PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4 % SDS (w/v), 20 % (v/v) glycerol, 144 mM 2-mercaptoethanol and 0.2 % (w/v) Bromophenol Blue] to prepare total trophozoite lysate. The other half was incubated in 5 % (w/v) sorbitol for 10 min to lyse trophozoites. The sample was centrifuged at 12000 ***g*** for 10 min at 4 °C and the supernatant (cytosol fraction) and pellet were separated. The pellet was washed three times with PBS, resuspended in PBS, subjected to three freeze/thaw cycles and centrifuged at 12000 ***g*** for 10 min at 4 °C. The supernatant was collected as the vacuolar fraction and the pellet as the membrane fraction. The concentration of each subcellular fraction and the total trophozoite lysate was adjusted to represent 5×10^6 parasites/μl in SDS/PAGE sample buffer and stored at −70 °C.

Amplification, cloning and sequence analysis of the falcipain-3 gene

A BLAST (basic local alignment search tool) search of the National Center for Biotechnology Information *P. falciparum* genome database identified a putative cysteine protease gene (*falcipain-3*) as most similar to *falcipain-2*. Primers (forward 5′-TTTTCAATTATTTTATTTTTTGTTTGAAC-3′; reverse 5′-TATTAAGGAAAATCTATTTTATTATTATG-3′) flanking the predicted open reading frame (ORF) were used to PCR-amplify the target sequence from W2-strain genomic DNA. This DNA had been purified from schizont-stage parasites by overnight lysis in a solution containing 10 mM Tris/HCl, 100 mM EDTA, 0.5 % (w/v) SDS and 100 μg/ml proteinase K (pH 8.0) at 37 °C and sequential extraction with phenol and phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), prior to precipitation of the DNA with propan-2-ol. The amplified fragment was purified using the Qiagen gel extraction kit and cloned into the pCR2.1-TOPO vector using the TA-cloning kit (Invitrogen). Plasmid DNA containing the PCR fragment (pTOPO-FP3) was purified from multiple clones using a Qiagen Miniprep kit and sequenced in both directions by dideoxy sequencing at the UCSF Biomolecular Resource Center. Sequence data were analysed using the DNASTAR program for alignment, the PRED-TMR algorithm and PSORT II for transmembrane segment determination [15,16].

Southern blotting

P. falciparum genomic DNA (10 μg/lane) was digested with restriction endonucleases, electrophoresed on a 0.7 % (w/v) agarose gel and transferred on to a nylon membrane (Amersham Pharmacia Biotech). A 222 bp fragment, corresponding to the 5′ region of *profalcipain-3*, was amplified from pTOPO-FP3 (forward 5′-GTTTGAACAATGGAATATCATATGGAATA TTCACCG-3′; reverse 5′-ATTTGATAAACTATTAGTAAACAAGGTCTTGTTATTTTCATTTCT-3′), purified using the Qiagen gel extraction kit and labelled with [α-^{32}P]dATP (Amersham Pharmacia Biotech) using the Random Primers DNA labelling system (Life Technologies). The membrane was prehybridized in 5 × SSPE [SSPE is 0.15 M NaCl/0.01 M NaH_2PO_4 (pH 7.4)/1 mM EDTA]/0.5 % (w/v) SDS/5 × Denhardt's solution for 5 h at 55 °C. Hybridization was carried out overnight at 65 °C in prehybridization buffer containing the probe. The membrane was washed twice at 25 °C for 5 min with 2 × SSC (SSC is 0.15 M NaCl/0.15M sodium citrate)/0.1 % (w/v) SDS, once at 55 °C for 15 min with 2 × SSC/0.1 % SDS (w/v) and once at 60 °C for 10 min with 0.5 × SSC/0.1 % SDS (w/v).

Isolation and analysis of RNA

For isolation of total RNA, stage-specific parasites (collected as described in [9]) were suspended in TRIZOL and processed as recommended by the manufacturer (Life Technologies). The RNA pellet was suspended in sterile water [treated with diethylpyrocarbonate ('DEPC')] and treated with DNase I (Boehringer Manheim) at 37 °C for 30 min. The purity and quantification of RNA were evaluated spectrophotometrically. Before the reverse transcriptase-PCR (RT-PCR), RNA was evaluated for genomic DNA contamination by PCR using primers specific for *falcipain-3*, 500 ng of RNA and the conditions used for PCR of cDNA. DNA-free RNA samples were reverse-transcribed using the Superscript First Strand Synthesis for RT-PCR (Life Technologies) as recommended by the manufacturer. Briefly, 10 μg of each RNA sample was annealed with the oligo$(dT)_{12–18}$ primer, extended with Superscript II RT at 42 °C for 70 min, incubated at 70 °C for 15 min and then subjected to RNase H treatment.

To investigate stage-specific transcription of *falcipain* genes, a portion of each *falcipain* gene (corresponding to the most C-terminal region of the prodomain and the entire mature domain) was amplified from each stage-specific *P. falciparum* cDNA. The primer pairs used (restriction enzyme sites are in bold type) were: *falcipain-1* (nucleotides 792–1721; forward 5′-GAATGCTAT**GGATCC**AAAGAAAGTAAATCAGTTTAG-3′; reverse 5′-TGGTT**AAGCTT**ACAAGATAGGATAGAAGAC-3′), *falcipain-2* (610–1463; forward 5′-ATAGTT**GGATCC**AAAAAAGAATTAAACAGATTTGCC-3′; reverse 5′-TGAC**AAGCTT**ATTCAATTAATGGAATGAATGCATCAGTACC-3′), and *falcipain-3* (616–1488; forward 5′-AACAAA**GGATCC**AATAGTTTATATAAAAGGGGTATG-3′ reverse 5′-TTA**GTCGAC**TTATTCAAGTAATGGTACATAAGCTTCTGTT-3′). Amplifications were carried out under identical conditions in 50 μl reaction mixtures [0.5 μM each primer, 250 μM each dNTP (Stratagene), 2 mM $MgCl_2$ and 2.5 units of *Taq* DNA polymerase (Life Technologies)] containing 0.5 μl (1 μl for *falcipain-1*) of cDNA template (diluted 1:40).

Expression, purification and refolding of recombinant falcipain-3

A fragment coding for the most C-terminal 33 amino acid residues of the prodomain and entire mature domain (−33FP3) was amplified from pTOPO-FP3 using Vent DNA polymerase (New England BioLabs) and primers containing *Bam*HI (forward 5′-AACAAA**GGATCC**AATAGTTTATATAAAAGGGGTATG-3′) and *Sal*I (reverse 5′-TTA**GTCGAC**TTATTCAAGTAATGGTACATAAGCTTCTGTT-3′) cleavage sites. The PCR product was digested with *Bam*HI and *Sal*I, gel purified and ligated into the expression vector pQE-30 (Qiagen) to generate pQE-FP3. This construct was used to transform M15(pREP4) *Escherichia coli*. pQE-FP3 DNA was isolated from multiple-expressing clones and the insert sequence was confirmed by dideoxy sequencing. Expression vector pQE-FP3 provided a His-tag at the N-terminus of recombinant falcipain-3, allowing one-step purification of the expression product by nickel-nitrilotriacetic acid (Ni-NTA; Qiagen) chromatography. Falcipain-3 was expressed from a positive clone upon induction with isopropyl β-D-thiogalactopyranoside ('IPTG'), inclusion bodies were prepared and falcipain-3 was purified from solubilized [in 6 M guanidine/HCl, 20 mM Tris/HCl, 500 mM NaCl and 10 mM imidazole (pH 8.0)] inclusion bodies by Ni-NTA chromatography, as described for falcipain-2 [17].

Refolding of Ni-NTA-purified falcipain-3 was optimized by testing over 200 buffer combinations in a systematic microdilution format as described previously [17]. Briefly, denatured-reduced falcipain-3 was diluted 100-fold (10 μg/ml, final concentration) in 300 μl of each ice-cold refolding buffer, followed by incubation at 4 °C for 20 h. For evaluation of refolding efficiencies, 20 μl of each refolding sample was incubated with assay buffer [100 mM sodium acetate (pH 5.5) and 10 mM dithiothreitol (DTT)] at 25 °C for 30 min. Z-Phe-Arg-AMC was added at a final concentration of 50 μM and hydrolysis was monitored as described below. After identification of the best microplate refolding conditions [100 mM Tris/HCl (pH 8.0), 1 mM EDTA, 20 % (w/v) sucrose, 250 mM L-arginine, 1 mM GSH and 0.5 mM GSSG], further testing identified the optimal refolding buffer [25 mM 3-(cyclohexylamino)propane-1-sulphonic acid ('CAPS'), 20 % (w/v) sucrose, 250 mM L-arginine, 1 mM EDTA, 1 mM GSH and 0.5 mM GSSG (pH 9.5)]. Large-scale refolding was carried out with a 100-fold dilution of 100 mg of reduced/denatured Ni-NTA-purified protein in 2 l of ice-cold refolding buffer at 4 °C for 20 h. The refolded sample was concentrated to 100 ml using a High-Performance Ultrafiltration Cell (Model 2000; Amicon) with a 10 kDa cut-off membrane.

For additional purification, the pH of the refolded sample was adjusted to 5.5 with 3.6 M sodium acetate (pH 2.6) and DTT was added to a final concentration of 5 mM. The precipitated material was removed using a 0.22 μm filter (Millipore) and the sample was incubated at 37 °C for 2 h to allow processing to the active enzyme. The pH was then readjusted to 6.5 with 1 M Tris/HCl (pH 8.0) and the protein was applied on to a Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 20 mM Bis-Tris/HCl (pH 6.5) (maintained at 4 °C). The column was washed with 5–10 bed volumes of the same buffer and the protein was eluted with a 0–0.4 M linear NaCl gradient in a solution of 20 mM Bis-Tris/HCl (pH 6.5) and 1 M NaCl over 30 min at a flow rate of 1.5 ml/min. Enzyme-containing fractions, monitored by hydrolysis of Z-Leu-Arg-AMC, were pooled and concentrated (10 kDa cut-off Centriprep; Millipore). An equal volume of glycerol was added and the enzyme was stored at −20 °C. For N-terminal sequencing of the mature enzyme, processed and purified falcipain-3 was electrophoresed and transferred on to an Immobilon-P^{SQ} membrane (Millipore) and stained with Coomassie Blue. The band corresponding to falcipain-3 was excised and sequenced at the UCSF Biomolecular Resource Center (San Francisco, CA, U.S.A.).

Production of specific antibodies

Antisera were raised against Ni-NTA-purified recombinant truncated-profalcipain-2 (−35FP2 [9]), falcipain-3 (−33FP3, prepared as described above) and the mature domain of falcipain-1 [8]. The recombinant proteins were separated by SDS/PAGE under reducing conditions on 12 % gels. The gels were stained with Coomassie Blue and washed extensively with PBS. Protein bands were then excised, emulsified in complete (day 0) or incomplete (days 14, 35 and 68) Freund's adjuvant and injected intraperitoneally into rats. Rats were sacrificed and their serum collected on day 82.

Affinity columns were prepared as follows. Recombinant antigens (10–20 mg each) were desalted on Sephadex-10 columns equilibrated with 100 mM sodium carbonate (pH 8.4) containing 500 mM NaCl and crosslinked to cyanogen bromide-activated Sepharose 4B beads in 2–5 ml of the same buffer overnight at 4 °C. The beads were washed extensively with the same buffer and blocked with 100 mM Tris/HCl (pH 8.0) followed by 1 M ethanolamine (pH 8.0). The beads were washed again with 500 mM NaCl in PBS and the affinity resins were stored in binding buffer (PBS, 0.1 % BSA and 0.01 % thimerosal) at 4 °C. For antibody purification, antisera were diluted 1:50 in binding buffer, passed twice over the appropriate antigen columns and washed extensively with 500 mM NaCl in PBS followed by 10 mM sodium phosphate buffer (pH 6.8) containing 150 mM NaCl. Antibodies were eluted with 3 ml of 100 mM glycine (pH 2.6) containing 150 mM NaCl and neutralized with 0.3 ml of 1 M Tris/HCl (pH 8.0) and 0.7 ml of binding buffer. Each stock of purified antibodies was passed over the other two antigen columns to eliminate cross-reactive antibodies and stored at 4 °C.

Immunoblotting

Recombinant proteins, parasite extracts, total trophozoite lysates, subcellular fractions and affinity purified protease fractions (prepared as previously described [9]) were separated by SDS/PAGE on 10 % (w/v) gels and transferred on to PVDF membranes. The membranes were blocked with 0.2 % I-block (Tropix), washed with PBS containing 0.1 % Tween-20 and incubated with antibody stocks (1:20000 dilution) for 1 h at 37 °C. The membranes were washed and incubated with alkaline phosphatase-conjugated goat anti-rat IgG (1:20000 dilution; Jackson Immunoresearch) at 37 °C for 1 h. After incubation, the membrane was washed again and the antigen–antibody complexes were developed using a Western-Star chemiluminescence kit as described by the manufacturer (Tropix).

Characterization of recombinant falcipain-3

Total protein concentration was determined using a Bio-Rad Protein Assay dye; active enzyme concentration was determined by active-site titration with Z-Phe-Arg-FMK. Substrate gel analysis was performed as previously described using SDS/PAGE under non-reducing conditions with gels co-polymerized with 0.1 % (w/v) gelatin [3]. Fluorometric assays of falcipain-3 activity were carried out in 350 μl of assay buffer, containing 45 nM enzyme, or with adjustments in reagents for particular experiments as described in the Figure legends. Fluorogenic substrates (routinely 50 μM Z-Phe-Arg-AMC or Z-Leu-Arg-AMC) were added and activity was monitored as the release of fluorescence (excitation 355 nm; emission 460 nm) over 30 min (15 min for pH activity profile) at 25 °C with a Labsystems Fluoroskan II spectrofluorometer. For the analysis of pH stability, enzymes (90 nM) were added to prewarmed (37 °C) buffer [1 mM GSH and 1 mM EDTA in either 50 mM sodium acetate (pH 4.0–6.0) or 50 mM sodium phosphate (pH 6.5–7.5)] and incubated at 37 °C. After appropriate time intervals, 30 μl aliquots were added to 320 μl of assay buffer containing 25 μM Z-Leu-Arg-AMC and activities were measured for 10 min as described above. For all assays, substrate concentrations were saturating within the time course studied, such that curves for fluorescence over time remained linear.

Enzyme kinetics

Rates of hydrolysis of varied concentrations of peptide-AMC substrates were determined at constant enzyme concentration (45 nM) in 350 μl of assay buffer at 25 °C. Enzyme activity was monitored as described above and K_m, V_{max}, and k_{cat} were determined using the Enzfitter program [18].

Hydrolysis of haemoglobin

Recombinant falcipain-3 and falcipain-2 (prepared as previously described [9]) were evaluated for their ability to hydrolyse native haemoglobin and acid-denatured globin [19]. For evaluation at

different enzyme concentrations, 30 μl reactions containing 100 mM sodium acetate (pH 5.5), 1 mM GSH and 3 μg of haemoglobin or globin were incubated at 37 °C for 2 h (1 h for globin) and stopped with 5 μl of non-reducing SDS/PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4 % (w/v), 20 % (v/v) glycerol and 0.2 % (w/v) Bromophenol Blue]. For evaluation at different time intervals, 300 μl reactions in assay buffer containing 1 mM GSH, 30 μg of native haemoglobin or globin and 400 nM enzyme (50 nM for globin hydrolysis) were incubated at 37 °C. Aliquots (30 μl) were withdrawn at indicated time intervals and the reactions were stopped as noted above. Reaction products were resolved by SDS/PAGE.

RESULTS

Molecular analysis of falcipain-3

A BLAST search of the available *P. falciparum* genome database identified a third putative cysteine protease sequence, *falcipain-3*, that was homologous to the previously described *falcipain-1* [7] and *falcipain-2* [9] genes. Primers flanking the predicted ORF were used to amplify *falcipain-3* from W2 strain genomic DNA. Sequencing of multiple-cloned fragments revealed an identical 1539 bp sequence with an uninterrupted ORF of 1476 bps flanked by stop codons. The first start codon of the ORF was chosen as the likely true translation initiation codon based on the first ATG rule [20,21], the presence of a lower eukaryotic consensus translation initiation sequence (AXXATGG; where X is any nucleotide) [20,22], and a highly AT rich upstream sequence, a feature of non-coding *P. falciparum* DNA [23]. The ORF encodes a fairly typical papain-family cysteine protease of 492 amino acids that is most similar, among known proteases, to falcipain-2 (53 % identity) and falcipain-1 (28 %). Falcipain-3 shares many features with falcipain-2, including (1) a 20 amino acid putative membrane-spanning domain 36 amino acids from the start methionine residue, a much larger pro-region than that of most papain-family proteases, (2) the presence of the ERFNIN and GNFD pro-sequence motifs, which are typical of cathepsin L-like cysteine proteases [24–26], (3) conservation of papain-family catalytic residues, (4) nine cysteine residues (predicting four disulphide bonds) in the mature protease sequence, and (5) an unusual insert between highly conserved amino acid residues near the C-terminus (Figure 1).

P. falciparum genomic DNA was digested with five restriction endonucleases and probed with a *falcipain-3* prodomain fragment that does not contain cleavage sites for any of the endonucleases. For each endonuclease, a single restriction fragment hybridized with the *falcipain-3* probe (Figure 2). In each case, the fragment was one of two previously recognized by a mature *falcipain-2* probe [9], confirming our prior hypothesis that the recognition of two fragments by that probe was due to hybridization with both *falcipain-2* and *falcipain-3*, and that both of these genes are single copy.

The stage-specific transcription of *falcipain-3* was compared with that of *falcipain-1* and *falcipain-2* using RT-PCR with RNA

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FP3  MEYHMEYSPNEVIKQERELVFVGKEKSGSKFKRKRSIFIVLTVSICFMFALMLFYFTRNEN  60
FP2  MDYNMDYAPHEVISQQGERFVDKYVDRKILKNKKSLLVIISLSVLSVVGFVLFYFTPNSR  60
Pap  M-----------------------------------AMIPSISKLLFVAICLFVYMG---  22

FP3  NKTLFTNSLSNNINDDYIINSLLKSESGKKFIVSKLEELISSYDKEKKMRTTGAEENNMN  120
FP2  KSDLFKNSSVENNNDDYIINSLLKSPNGKKFIVSKIDEALSFYDSKKNDINKYNEGNN-N  119
Pap  ------------------------------------------------------------

FP3  MNGIDDKDNKSVSFVNKKNGNLKVNNNNQVSYSNLFDTKFLMDNLETVNLFYIFLKENNK  180
FP2  -N---NADFKGLSLF-KENTPSNNFIHNKDYFINFFDNKFLMNNAEHINQFYMFIKTNNK  174
Pap  ------LSFGDFSIVG-------------------YSQNDLTSTERLIQLFESWMLKHNK  57

FP3  KYETSEEMQKRFIIFSENYRKIELHNKKTNSLYKRGMNKFGDLSPEEFRSKYLNLKTHGP  240
FP2  QYNSPNEMKERFQVFLQNAHKVNMHNNNKNSLYKKELNRFADLTYHEFKNKYLSLRSSKP  234
Pap  IYKNIDEKIYRFEIFKDNLKYIDETNKKNNS-YWLGLNVFADMSNDEFKEKYTGSIAGN-  115

FP3  FKTLSPPVSYEANYEDVIKKYKPADAKLDRIAYDWRLHGGVTPVKDQALCGSCWAFSSVG  300
FP2  LKN-SKYLLDQMNYEEVIKKYR-GEENFDHAAYDWRLHSGVTPVKDQKNCGSCWAFSSIG  292
Pap  ------YTTTELSYEEVLNDGDVNIPEY----VDWRQKGAVTPVKNQGSCGSCWAFSAVV  165

FP3  SVESQYAIRKKALFLFSEQELVDCSVKNNGCYGGYITNAFDDMIDLGGLCSQDDYPYVSN  360
FP2  SVESQYAIRKNKLITLSEQELVDCSFKNYGCNGGLINNAFEDMIELGGICPDGDYPYVSD  352
Pap  TIEGIIKIRTGNLNEYSEQELLDCDRRSYGCNGGYPWSALQLVAQYG-IHYRNTYPYEGV  224

FP3  LPETCNLKR---CNERYTIKSYVSIPDDKFKEALRYLGPISISI-AASDDFAFYRGGFYD  416
FP2  APNLCNIDR---CTEKYGIKNYLSVPDNKLKEALRFLGPISISV-AVSDDFAFYKEGIFD  408
Pap  QRY-CRSREKGPYAAKTDGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGIFV  283

FP3  GECGAAPNHAVILVGYGMKDIYNEDTGRMEKFYYYIIKNSWGSDWGEGGYINLETDENGY  476
FP2  GECGDQLNHAVMLVGFGMKEIVNPLTKKGEKHYYYIIKNSWGQQWGERGFINIETDESGL  470
Pap  GPCGNKVDHAVAAVGYGPN--------------YILIKNSWGTGWGENGYIRIKRGTGNS  329

FP3  KKTCSIGTEAYVPLLE  492
FP2  MRKCGLGTDAFIPLIE  484
Pap  YGVCGLYTSSFYPVKN  345
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Figure 1 Sequence alignment

The deduced amino acid sequences of falcipain-3 (FP3), falcipain-2 (FP2) and papain (Pap) were aligned by the DNASTAR program using the CLUSTAL method [36]. Identities with falcipain-3 are shaded, predicted transmembrane domains are boxed, amino acids representing the ERFNIN and GNFD prodomain motifs are labelled with asterisks, the positions of mature domain processing sites are indicated by arrowheads, and catalytic amino acids are highlighted.

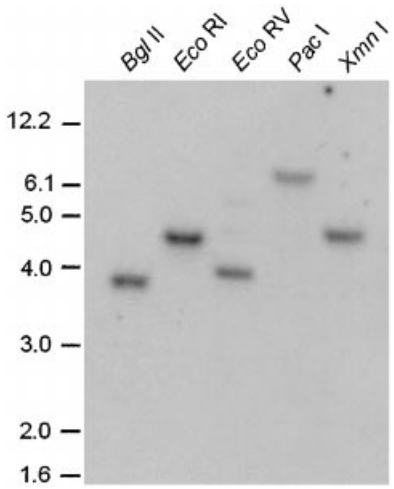

Figure 2 Southern-blot analysis of falcipain-3

P. falciparum genomic DNA (10 μg/lane) was digested by the restriction endonucleases shown, resolved on a 0.7% agarose gel, transferred on to a nylon membrane, hybridized with a falcipain-3 probe and visualized by autoradiography. The positions of size markers (in kbp) are shown on the left.

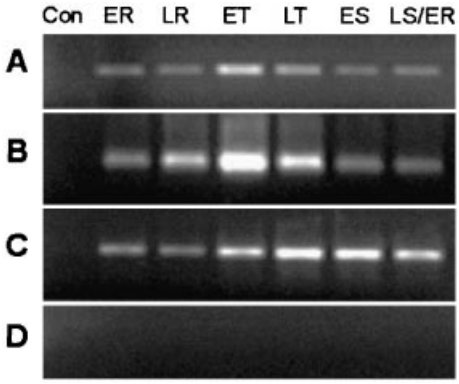

Figure 3 Stage-specific transcription of *falcipain* genes

PCR was performed with stage-specific cDNA and primers specific for *falcipain-1* (**A**), *falcipain-2* (**B**) and *falcipain-3* (**C**, **D**). Reactions were performed under identical conditions, using equal quantities of reverse-transcribed RNA for each reaction, except that, to allow resolution of products of low-yield reactions, twice the quantity of cDNA template was used for *falcipain-1* reactions and twice the volume of each *falcipain-1* reaction was electrophoresed. Products amplified from early rings (ER), late rings (LR), early trophozoites (ET), mid-late trophozoites (LT) early-mid schizonts (ES) and late schizonts/early rings (LS/ER) were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. Controls included amplifications using RNA templates (**D**) and no template (Con).

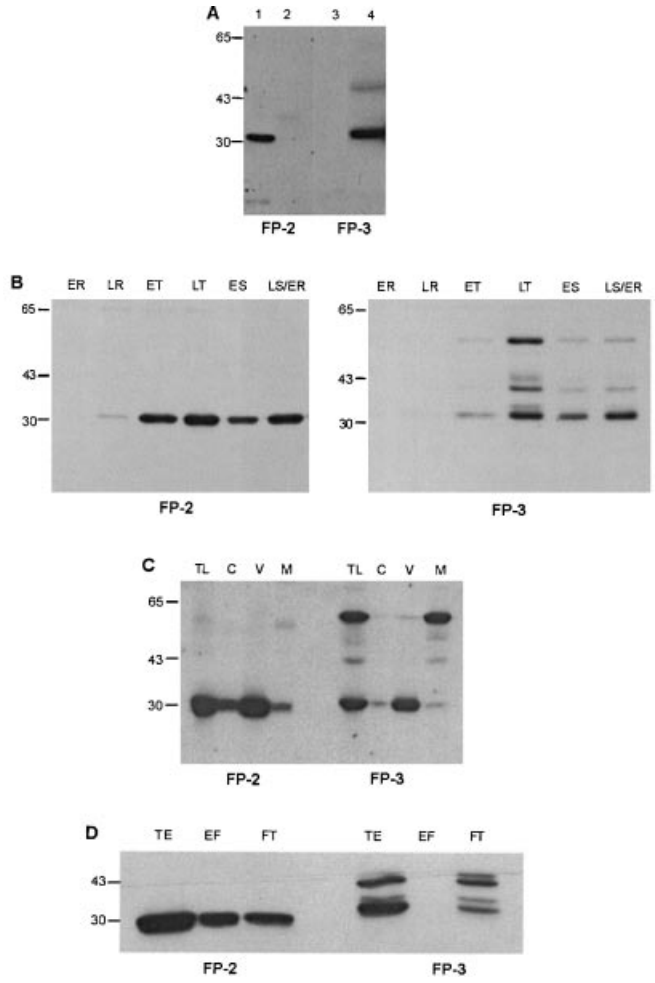

Figure 4 Immunoblot analysis of falcipain-2 and falcipain-3

In each of the immunoblots, proteins were resolved by SDS/PAGE under reducing conditions on 10% gels, transferred on to PVDF membranes and the membranes were probed with affinity purified antibodies directed against falcipain-2 (FP-2) and falcipain-3 (FP-3). The positions of molecular-mass markers (in kDa) are shown on the left. (**A**) Specificity of antisera. Recombinant falcipain-2 (lanes 1 and 3) and falcipain-3 (lanes 2 and 4) were probed with antibodies against the two proteins as labelled. (**B**) Stage specificity of falcipain expression. Extracts of synchronized parasites, corresponding to 1.8×10^7/lane and labelled as in Figure 3, were probed. (**C**) Presence of falcipains in different cellular fractions. Total trophozoite lysate (TL), cytosol (C), vacuolar (V) and membrane (M) fractions, each corresponding to 5×10^7 parasites/lane were prepared as described and probed. (**D**) Presence of falcipains in purification fractions. Starting trophozoite extract (TE), the eluted fraction (EF) that bound and then was eluted from a Sepharose-Gly-Phe-Gly-semicarbazone column and the flow-through (FT) material, that did not bind to column, were probed.

collected from highly synchronous parasites. Amplification of target sequences from cloned genes and genomic DNA indicated that the falcipain primers were each specific for their respective targets and that, under the conditions used, they amplified equivalent quantities of DNA (results not shown). In the RT-PCR analysis, primers for all three plasmodial proteases amplified cDNA from erythrocytic parasites (Figure 3). Maximum transcription of *falcipain-1* and *falcipain-2* occurred in early trophozoites, consistent with results from previous Northern-blot analyses [7,9]. Transcription of *falcipain-3* peaked in late trophozoites and early schizonts.

Comparison of the expression of falcipain-2 and falcipain-3 in erythrocytic parasites

Polyclonal antisera were raised in rats against the three recombinant falcipains. Immunoblots showed that the affinity-purified antibodies were each specific for their respective recombinant antigens (Figure 4A). Erythrocytic *P. falciparum* parasites develop over 48 h from ring to trophozoite to schizont stages. Immunoblots of parasite extracts from different erythrocytic stages detected only a very weak falcipain-1 signal in trophozoites (results not shown), as seen previously [7], but strong reactivity with falcipain-2 and falcipain-3 in trophozoites and schizonts (Figure 4B). Falcipain-2 was prominent in early and late trophozoites, whereas falcipain-3 did not reach maximal levels

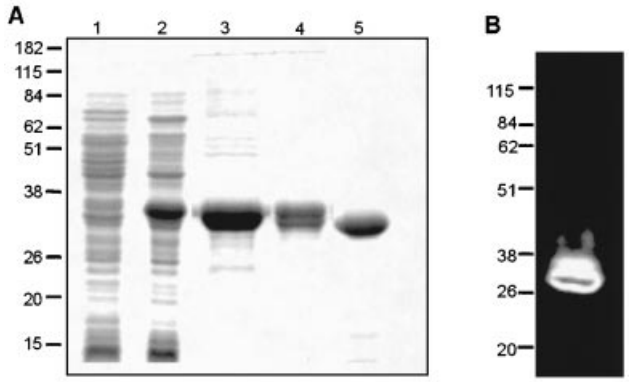

Figure 5 Expression, purification and refolding of recombinant falcipain-3

(**A**) Truncated profalcipain-3 (—33FP3) was expressed in M15(pREP4) *E. coli*, uninduced (lane 1) and induced cells (lane 2), purified by Ni-NTA chromatography (lane 3), refolded (lane 4), processed into the active enzyme and further purified by anion-exchange chromatography (lane 5). Each sample was resolved by SDS/PAGE under reducing conditions on a 12% gel and the gel was stained with Coomassie Blue. (**B**) The refolded processed protein, corresponding to lane 5, was also evaluated by SDS/PAGE on a 12% gelatin substrate. The position of molecular-mass markers (in kDa) are shown on the left in each panel.

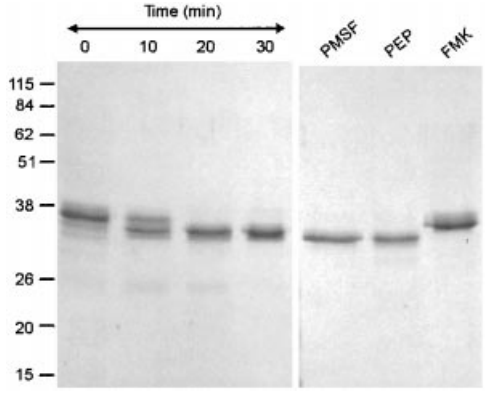

Figure 6 Processing of recombinant falcipain-3

Refolded falcipain-3 was incubated in 100 mM sodium acetate (pH 5.5) and 5 mM DTT in the absence of inhibitors for 0–30 min or in the presence of either PMSF, pepstatin (PEP) or Z-Phe-Arg-FMK (FMK) for 2 h. Proteins were resolved by SDS/PAGE under reducing conditions on 12% gels and stained with Coomassie Blue. The positions of molecular mass-markers (in kDa) are shown on the left.

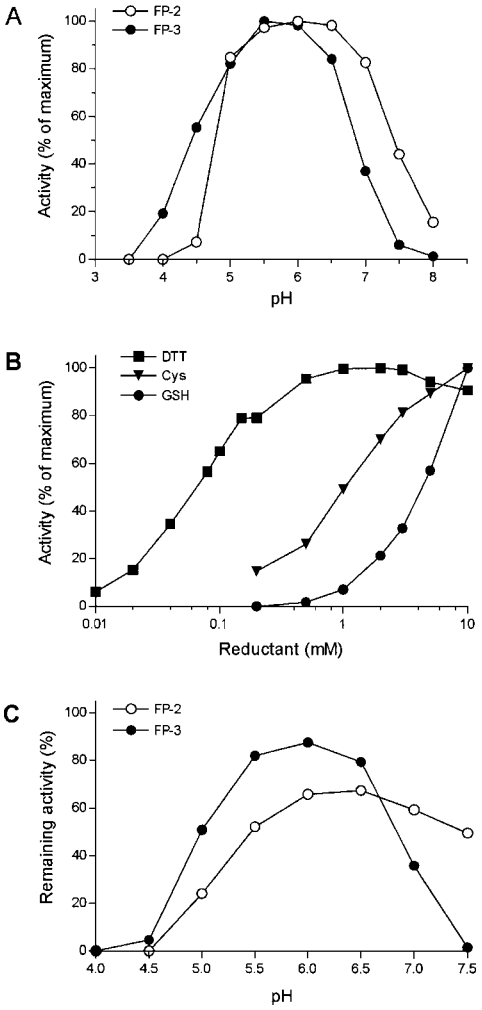

Figure 7 Biochemical features of falcipain-3

The hydrolysis of Z-Leu-Arg-AMC by falcipain-3 (FP-3) and falcipain-2 (FP-2) at different pHs [in 100 mM sodium acetate (pH 3.5–6.0) or sodium phosphate (pH 6.5–8)] (**A**) and with different reductants [DTT, L-cysteine (Cys) and GSH; FP-3 only] (**B**) were assayed as described in the Experimental section. Results are expressed as the percentage of maximum activity for each assay. (**C**) The pH stability of the recombinant enzymes was compared by incubating them (90 nM) in 1 mM EDTA and 1 mM GSH in either 50 mM sodium acetate (pH 4.0–6.0) or sodium phosphate (6.5–7.5) at 37 °C for 3 h. Aliquots of equal volume were assayed for the hydrolysis of 25 μM Z-Leu-Arg-AMC in assay buffer and compared with the activity of control samples that were not incubated in buffer.

until the late trophozoite stage. Anti-falcipain-3 antibodies recognized a number of species in parasite extracts, consistent with the presence of abundant proform and processing intermediates in trophozoites and schizonts, whereas, for falcipain-2, only the mature protease was recognized.

To investigate the subcellular distribution of the proteases, total parasite lysates and cytosol, vacuole and membrane fractions of trophozoite-stage parasites were evaluated in immunoblots. Abundant mature falcipain-2 and falcipain-3 were detected in total lysates and the vacuole fraction (Figure 4C). Much less of the mature enzymes were identified in the cytosol fraction (which may have been contaminated with material that leaked from vacuoles during sample processing). In contrast to the pattern for the mature proteases, a prominent falcipain-3 proform and a small quantity of a falcipain-2 proform were present only in the membrane fraction. Thus mature falcipain-2 and falcipain-3 are located primarily in a vacuolar fraction, most likely the parasite food vacuole, and proforms of the proteases are membrane-associated. The proteases differed in that the falcipain-3 proform was much more abundant.

The identification of abundant falcipain-3 in trophozoites was difficult to reconcile with our prior identification of falcipain-2 as the only cysteine protease purified from trophozoites with affinity chromatography using a Sepharose-Gly-Phe-Gly-semicarbazone column [9]. To address this question, we performed the previously described purification with this ligand and examined the chromatography fractions by immunoblotting (Figure 4D). Anti-falcipain-2 antibodies recognized protein that bound to the ligand and unbound material, anti-falcipain-3 antibodies recognized only unbound material and anti-falcipain-1 did not recognize any protein (results not shown). Thus falcipain-3 was

Table 1 Kinetic parameters for substrate hydrolysis by falcipain-3 and falcipain-2

Values are means from a representative experiment carried out in duplicate. Data for recombinant falcipain-2 are from [9]. Substrates not cleaved by falcipain-3: H-Ala-Arg-AMC, Bz-Val-Gly-Arg-AMC, Boc-Val-Gly-Arg-AMC, Boc-Leu-Gly-Arg-AMC, Boc-Gly-Pro-Arg-AMC, Boc-Val-Pro-Arg-AMC, Boc-Gln-Ala-Arg-AMC and Z-Gly-Gly-Arg-AMC. Abbreviations used: Bz-, benzoyl; Boc-, t-butyloxycarbonyl.

Substrates	Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)
Z-Phe-Arg-AMC	FP-3	71.3	0.009	120
	FP-2	9.1	0.412	45100
Z-Leu-Arg-AMC	FP-3	72.0	0.043	590
	FP-2	8.4	0.884	106000
Z-Val-Arg-AMC	FP-3	185.0	0.005	26
	FP-2	59.6	0.324	5440
Z-Val-Leu-Arg-AMC	FP-3	27.4	0.062	2,240
	FP-2	3.1	0.190	61900
Z-Val-Val-Arg-AMC	FP-3	39.8	0.008	199
	FP-2	6.7	0.027	4030
Boc-Val-Leu-Lys-AMC	FP-3	10.9	0.007	623
	FP-2	3.4	0.167	49700

not previously identified because it did not bind to the affinity ligand, and the 5–10% of cysteine protease activity (measured against the Z-Phe-Arg-AMC substrate) that did not bind to the ligand [9] consisted of falcipain-3, unbound falcipain-2, and possibly other proteases.

Expression, refolding and processing of falcipain-3

A DNA fragment coding for the most C-terminal 33 amino acid residues of the prodomain and entire mature domain of falcipain-3 (−33FP3) was amplified, ligated into the pQE-30 vector and used to transform M15(pREP4) *E. coli*. Recombinant falcipain-3 was expressed as an insoluble protein, purified from inclusion bodies by Ni-NTA chromatography under denaturing conditions and refolded in an alkaline buffer (Figure 5). Optimization of refolding conditions utilized a systematic microdilution approach as previously described [17]. Refolded falcipain-3 was processed to a smaller enzymically active species of the size predicted for mature falcipain-3 upon exposure to an acidic buffer (Figure 6). Based on comparison of dye quantitation and active-site titration results, 43% of recombinant falcipain-3 was enzymically active. The requirement for acidic pH for the efficient processing of falcipain-3 contrasted with results for falcipain-2, which was rapidly processed during refolding at alkaline pH [9]. In both cases, processing was autocatalytic as it was blocked by inhibitors of cysteine proteases, but not by those of other proteolytic classes (Figure 6). N-terminal sequencing of processed falcipain-3 revealed a single cleavage site between Lys^{242} and Thr^{243} (Figure 1).

Biochemical features of falcipain-3

Falcipain-3 had typical properties of a papain-family cysteine protease, including an acidic pH optimum, requirement for a reducing environment for maximum activity (Figure 7) and inhibition by standard cysteine protease inhibitors (results not shown). Although the enzymes both demonstrated acidic pH optima, falcipain-3 was considerably less active than falcipain-2 at pH 7.0–8.0, but more active at pH 4.0–4.5 (Figure 7A). A comparison of the pH stability showed that falcipain-3 was more stable at moderately acidic pH, and less stable at neutral pH than was falcipain-2 (Figure 7C). Evaluation of the hydrolysis of typical papain-family peptide substrates revealed marked differences between falcipain-2 and falcipain-3 (Table 1). Both enzymes preferred substrates with Leu at the P_2 position, and the enzymes shared the P_2 rank order of Leu > Phe > Val. However, for all tested substrates k_{cat}/K_m was markedly lower for falcipain-3, due both to higher K_m and lower k_{cat} values. For falcipain-3, but not falcipain-2, inclusion of a P_3 Val increased k_{cat}/K_m 4–8-fold, indicating a role for P_3 interactions in optimizing catalytic activity.

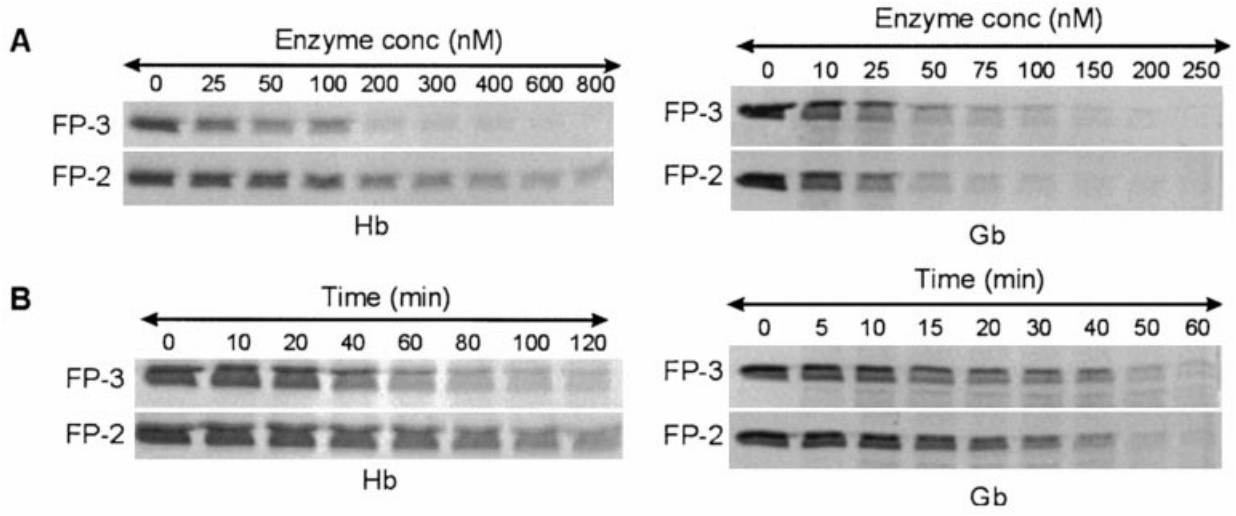

Figure 8 Hydrolysis of haemoglobin and globin

(**A**) Indicated concentrations of falcipain-2 (FP-2) or falcipain-3 (FP-3) were incubated with 3 μg of native haemoglobin (Hb) or 3 μg of globin (Gb) in 100 mM sodium acetate (pH 5.5) containing 1 mM GSH at 37 °C, for 2 h (1 h for globin). (**B**) Equal concentrations of the enzymes (400 nM and 50 nM for the Hb and Gb assays respectively) were incubated with 30 μg of Hb or Gb in 100 mM sodium acetate (pH 5.5) containing 1 mM GSH at 37 °C, and aliquots were taken at the indicated time points. The reactions were stopped, the proteins resolved by SDS/PAGE on 15% gels and the gels were stained with Coomassie Blue.

Table 2 Hydrolysis of haemoglobin and globin by falcipain-2 and falcipain-3 at various GSH concentrations

Reactions were carried out at 37 °C with 400 nM falcipain-2 and falcipain-3 for haemoglobin reactions and 50 nM of the enzymes for globin reactions in 100 mM sodium acetate (pH 5.5) containing various concentrations of GSH (0.1–10 mM). Samples were removed at various time points and analysed as described in Figure 8(B). The remaining substrate on the Coomassie Blue-stained gels was quantified by densitometry and compared with that at the start of each reaction. Values are from a single representative experiment and show the time (min) required for falcipain-2 and falcipain-3 to bring about 50% hydrolysis of haemoglobin and globin.

	Falcipain-2		Falcipain-3	
GSH (mM)	Haemoglobin	Globin	Haemoglobin	Globin
0.1	> 120	50	> 120	49
0.2	> 120	40	120	48
0.5	102	34	48	46
1.0	85	32	46	43
2.0	46	34	28	33
5.0	28	28	16	27
10.0	18	20	15	23

Haemoglobin hydrolysis by falcipain-3

The presumed principal natural substrate for falcipain-3 is haemoglobin, as large quantities of this protein must be hydrolysed by erythrocytic parasites [1,2]. Haemoglobin hydrolysis is probably a co-operative process, including cysteine, aspartic and metalloprotease activities, but a detailed characterization of this process is not yet available. We recently showed that recombinant falcipain-2 degraded native haemoglobin under acidic and mildly reducing conditions likely to be present in the food vacuole, and that denatured globin was cleaved much more readily than was haemoglobin [9]. To gain insight into the relative roles of falcipain-2 and falcipain-3 in haemoglobin hydrolysis, we compared their activities against haemoglobin and globin. Interestingly, although falcipain-3 was much less active than falcipain-2 against the tested peptide substrates, it was more active against native haemoglobin (Figure 8 and Table 2). At physiological concentrations of GSH (0.5–2.0 mM), hydrolysis of native haemoglobin was about twice as rapid for falcipain-3 as for falcipain-2. Both enzymes degraded globin much more readily than native haemoglobin, and the rate of hydrolysis of globin was similar for the two proteases (Figure 8 and Table 2).

The above analysis suggests that falcipain-3 is a more efficient haemoglobinase than falcipain-2, but, since the proteases have markedly different activities against peptide substrates, their relative abundance was uncertain. We therefore quantified the two enzymes in trophozoites using a quantitative immunoblotting technique. Known quantities of the two recombinant enzymes were immunoblotted and quantities of the proteases in late trophozoites were determined using the immunoblots of recombinant proteins as standards. Densitometric assessment led to the estimate that the concentration of falcipain-2 in trophozoites is 1.8 times that of falcipain-3. As falcipain-3 appears to cleave native haemoglobin about twice as rapidly as falcipain-2, the relative contributions of the two enzymes to the hydrolysis of native haemoglobin appear to be similar.

DISCUSSION

We have identified and characterized the *P. falciparum* cysteine protease falcipain-3. This enzyme is the second *P. falciparum* cysteine protease that has been definitively characterized as a trophozoite haemoglobinase and thus is another important potential drug target. The relative importance of falcipain-3 was initially underappreciated, as it has much less activity against commonly used peptide substrates than the previously described haemoglobinase, falcipain-2. However, on a molar basis falcipain-3 is a more effective haemoglobinase than falcipain-2, and both proteases rapidly hydrolyse globin to small fragments. Since the mature proteases are present at similar abundance in trophozoites, they are both likely to play key roles in the hydrolysis of haemoglobin to free amino acids.

Although falcipain-3 is very similar to falcipain-2, with 65% identity of mature protease domains, the enzymes have important differences, which suggest differences in their biological roles. The stability and activity of falcipain-3 are greater at acid pH and less at neutral pH than are those of falcipain-2. These differences are likely to explain the fact that specific antibodies recognized abundant proforms of falcipain-3, but not falcipain-2, in trophozoites. Falcipain-3 is apparently not as readily processed until it is transported to the acidic food vacuole. The pH stability and activity of falcipain-3 suggest that this protease is particularly suited for activity in the food vacuole, where haemoglobin is degraded. In addition, falcipain-3 was the more active cysteine protease against native haemoglobin, further suggesting that this protease plays a key role in the initial cleavages of haemoglobin that have previously been shown to be blocked by cysteine protease inhibitors [3,27]. The greater activity and stability of falcipain-2 at neutral pH suggest that this protease may have roles in addition to haemoglobin hydrolysis. Of interest in this regard is the observation that cysteine protease inhibitors block the release of merozoites from mature schizont-infected erythrocytes and thus halt parasite development [28–30]. In addition, a neutral cysteine protease activity [31] and recombinant falcipain-2 [32] were recently shown to cleave the erythrocyte cytoskeletal proteins, ankyrin and protein 4.1, at neutral pH. Thus falcipain-2 may act against both haemoglobin and erythrocyte proteins under different conditions. Similar diverse roles have been proposed for the *P. falciparum* aspartic protease, plasmepsin II, which cleaves haemoglobin at acidic pH and spectrin at neutral pH [33]. Indeed, both falcipain-2 and falcipain-3 are present through most of the erythrocytic life cycle and so both proteases may perform a number of functions. Falcipain-1 is also present in trophozoites, albeit apparently at much lower levels than the other two falcipains. Although recombinant falcipain-1 was shown to be capable of haemoglobin hydrolysis [8], its biological role is unknown. A BLAST search of the available *P. falciparum* genome database identified no other falcipain-like sequences, although a number of more distantly related putative papain-family genes were present.

The three falcipains and their known analogues from other plasmodial species make up a family within the papain superfamily with a number of unique features. These enzymes all have unusual prosequences that are 2–3 times the length of those of papain and most related enzymes. The C-terminal regions of falcipain prodomains are quite well (> 30%) conserved with papain, but the N-terminal regions are unique. The falcipain prosequences all contain a putative type II membrane-spanning domain as defined by a hydrophobic stretch of 17–20 amino acids preceded by multiple positively charged residues [34]. Interestingly, unusually long proforms and similarly conformed membrane-spanning sequences are also present in the *P. falciparum* aspartic proteases, plasmepsin I and plasmepsin II, and these proteins have been shown to insert into membranes [35].

Our results support a model [5] in which plasmodial haemoglobinases are delivered to the parasite plasma membrane by the

secretory pathway and then directed to the food vacuole as components of cytosolic vesicles. This model additionally offers the intriguing possibility that the active domains of these enzymes may, as predicted for type II membrane proteins, extrude from the plasma or parasitophorous vacuole membrane, thus allowing action against erythrocyte cytoskeletal proteins. Falcipain-2, which is more readily processed at neutral pH, is more likely to be activated in extravacuolar compartments, although our data suggest that most of the enzyme is transported to the food vacuole. Falcipain-3 is less active and less capable of autohydrolysis at neutral pH and thus, this enzyme is likely to be activated only after it reaches an acidic intracellular compartment. This suggestion is supported by the identification in trophozoites of only small quantities of falcipain-2 proforms, but abundant membrane-associated proforms of falcipain-3. In any event, our model suggests that a unique prosequence signalling system may both target plasmodial proteases to the food vacuole and deliver them to the proximity of erythrocyte cytoskeletal targets.

The characterization of falcipain-3 will aid in the full description of the mechanism of haemoglobin hydrolysis by malaria parasites. In addition, it highlights an unforseen level of complexity that should impact on drug discovery projects directed against plasmodial cysteine proteases. Since it appears that falcipain-2 and falcipain-3 are both key haemoglobinases, drug discovery efforts should take into account the inhibition of both enzymes. Indeed, it is likely that potent inhibition of both falcipain-2 and falcipain-3 will be required to effectively inhibit parasite haemoglobin hydrolysis and thereby block parasite development.

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