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Falcipain-1, a *Plasmodium falciparum* Cysteine Protease with Vaccine Potential▽

Amit Kumar,† Krishan Kumar, Reshma Korde, Sunil Kumar Puri,‡ Pawan Malhotra,* and Virander Singh Chauhan*

International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

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Cysteine proteases (falcipains) of *Plasmodium falciparum* are potential targets for antimalarial chemotherapy, since they have been shown to be involved in important cellular functions such as hemoglobin degradation and invasion/rupture of red blood cells during parasite life cycle. The role of falcipain-1 at the asexual blood stages of the parasite still remains uncertain. This is mainly due to a lack of methods to prepare this protein in an active form. In order to obtain biologically active falcipain-1, a number of falcipain-1 constructs were designed and a systematic assessment of the refolding conditions was done. We describe here the expression, purification, and characterization of a falcipain-1 construct encoding mature falcipain-1 and 35 amino acids from the C-terminal of the pro domain. Recombinant falcipain-1 was overexpressed in the form of inclusion bodies, solubilized, and purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography under denaturing conditions. A systemic approach was then followed to optimize refolding parameters. An optimum refolding condition was obtained, and the yield of the purified refolded falcipain-1 was ~1 mg/liter. Activity of the protein was analyzed by fluorometric and gelatin degradation assays. Immunolocalization studies using anti-falcipain-1 sera revealed a distinct staining at the apical end of the *P. falciparum* merozoites. Previous studies using falcipain-1-specific inhibitors have suggested a role of falcipain-1 in merozoite invasion. Based on its localization and its role in invasion, we analyzed the immunogenicity of falcipain-1 in mice, followed by heterologous challenge with *Plasmodium yoelii* sporozoites. Our results suggest a possible role of falcipain-1 in merozoite invasion.

Malaria is a one of the most important infectious diseases caused by protozoan parasites of the genus *Plasmodium*. The most virulent human malaria parasite, *Plasmodium falciparum*, is responsible for the death of 1.5 to 2.7 million people worldwide annually (3). With the emergence of insecticide-resistant mosquitoes and drug-resistant parasites, there is an urgent need to identify new drug targets and develop new antimalarials (26). Malarial proteases are attractive antimalarial targets because of their crucial role in parasite development, especially in the process of host erythrocyte rupture/invasion and hemoglobin degradation (27). Data mining approaches have identified a large number of putative proteases encoded in the *P. falciparum* genome (48). Depending upon their mode of action, these proteases have been classified into five different groups (aspartic, cysteine, serine, metallo-, and threonine proteases). Among these different proteases, the roles of cysteine proteases (falcipains) and aspartic proteases (plasmepsins) have been best characterized through the use of their specific inhibitors (2, 8, 19, 20, 36).

Four falcipains that have been identified so far in the *P. falciparum* genome are falcipain-1 (FP-1), falcipain-2 and -2′ (now known as FP-2A and -2B), and falcipain-3 (FP-3). Falcipain-2A, -2B, and -3 have been shown to be involved in hemoglobin degradation (37, 40, 41). A recent knockout study of falcipain-2A suggested that falcipain-2B takes over the functions of falcipain-2A in the knockout parasites (39). However, the physiological role of falcipain-1 in asexual blood stages of the parasite still remains uncertain. Salas et al. (33) and Malhotra et al. (17) have suggested that falcipain-1 acts as a hemoglobinase. Later, Greenbaum et al. (10) showed that falcipain-1 is active during the invasive merozoite stage, and falcipain-1 specific inhibitors blocked parasite invasion of host erythrocyte but had no effect on the parasite hemoglobin degradation activity (10). Recently, two independent knockout studies have suggested a role of falcipain-1 in oocyst production but not in erythrocyte development (7, 38).

Despite being the first cysteine protease to be discovered in *Plasmodium*, falcipain-1 has still not been well characterized. Difficulties in production of recombinant protease have prevented its detailed biochemical and functional characterization. Although Salas et al. (33) were able to produce falcipain-1 in soluble form using a baculovirus expression system, characterization of the recombinant protein was hampered by low yield and the simultaneous expression and interference of baculoviral cysteine proteases (33). More recently, FP-1 expressed as a fusion protein with maltose-binding protein (MBP) in *Escherichia coli* was once again obtained in low yields (9). Interestingly, the above two recombinant falcipain-1 preparations showed dissimilar activities; the baculovirus preparation was found to be active against the fluorogenic peptide substrate benzyloxycarbonyl-Phe-Arg-7-amino-4-methyl-cou-

* Corresponding author. Mailing address: International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India. Phone: 91 11 26189358. Fax: 91 11 26162316. E-mail for Virander Singh Chauhan: virander@icgeb.res.in. E-mail for Pawan Malhotra: pawanm@icgeb.res.in.

† Present address: 128 Polk Hall, NCSU, Raleigh, NC 27695.

‡ Present address: CDRI, Chattar Manzil Palace, Lucknow, UP 226001, India.

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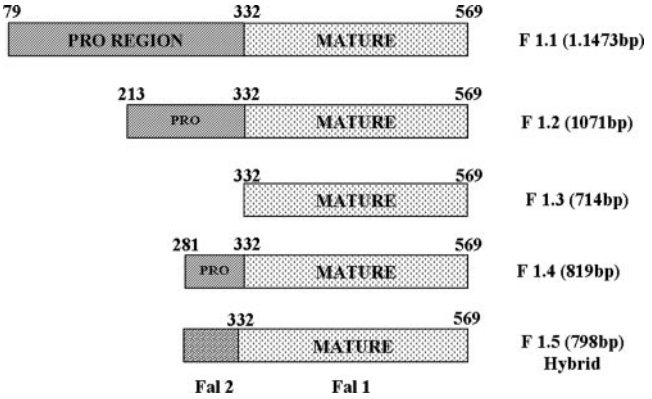

FIG. 1. Schematic representation of falcipain-1 constructs. The constructs vary with respect to the length of the pro regions: 253 amino acids for F1.1, 119 amino acids for F1.2, no pro region for F1.3; 42 amino acids for F1.4, and 28 amino acids of the pro region of falcipain-2 for F1.5.

marin (Z-F-R-AMC) at acidic pH, along with having hemoglobin degradation activity, while the MBP-fused falcipain-1 was active at neutral pH against the same peptide substrate but showed no hemoglobin degradation activity.

High-level expression of a protein is a prerequisite for structural and functional characterization studies. We therefore designed a number of falcipain-1 constructs, analyzed them for high-level expression in *E. coli*, and optimized the refolding conditions. One of the refolded proteins was tested for its vaccine potential using a heterologous *Plasmodium yoelii* mouse challenge model.

MATERIALS AND METHODS

Materials. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). *Taq* polymerase was from Bangalore Genei (Bangalore, India). Mouse anti-His sera, Ni^{2+}-nitrilotriacetic acid (NTA) agarose, pQE30 plasmid DNA, and M15 cells were from QIAGEN, Hilden, Germany. Isopropyl-β-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT), Q-Sepharose, and Superdex 75 column matrices for gel filtration purposes were acquired from Amersham Pharmacia. Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Coomassie brilliant blue R-250, horseradish peroxidase-conjugated anti-mouse secondary immunoglobulin G (IgG), and 4′,6′-diamidinophenylindole (DAPI) were purchased from Sigma, St. Louis, MO.

Cloning and expression of falcipain-1 constructs. Five different constructs, i.e., F1.1 (1.7 kb), F1.2 (1.1 kb), F1.3 (714 bp), F1.4 (819 bp), and F1.5 (798 bp), encoding the mature region and different lengths of the pro domain regions of falcipain-1 gene were designed (Fig. 1). DNA fragments coding for these constructs were amplified from genomic DNA of *P. falciparum* by using different primer sets. The full-length falcipain-1 gene was amplified using primers A (forward, 5′ATG GTT GCC ATA AAA GAA ATG 3′) and B (reverse, 5′ CCC **AAG CTT** CAA GAT AGG ATA GAA GAC 3′), the F1.1 gene fragment was amplified using primers C (forward, 5′ CC**G AAT TC**G GAA TTA CTT CGC GTT CTT TTA 3′) and B (reverse), gene fragment F1.3 was amplified using primers D (forward, 5′ CCG **GAA TTC** GGT ACC TGA AAT ATT AGA T3′) and B (reverse), and for F1.4 PCR amplification was done using primers E (forward, 5′ GAT C**GG ATC C**AT AGA AAA ATA TTC GAA A 3′) and B (reverse). The amplified falcipain-1 fragments were cloned in pGEM-T vector and excised using the respective endonucleases (restriction sites are shown in boldface), and the excised fragments were cloned in a number of *E. coli* expression plasmids (pET22b, pET32b, and pQE30) digested with the corresponding endonucleases. The pET22b and pET32b constructs were transformed into BL21(DE3) and AD494(DE3) *E. coli* cells, while pQE30 constructs were transformed into M15(pREP4) *E. coli* cells. To express F1.2 protein, the gene fragment was excised from the full-length falcipain-1 construct with BamHI and HindIII sites and cloned into pQE30 vector digested with the corresponding enzymes. The hybrid construct F1.5 was generated by cloning the mature region of falcipain-1 in the falcipain-2-expressing pQE30 clone described previously by Kumar et al. (14) at EcoRI and HindIII sites. The final F1.5 construct thus contained 28 amino acids from the pro domain of falcipain-2 and the mature region of falcipain-1. The constructs F1.4 and F1.5 were transformed into M15(pREP4) cells, while the F1.2 construct was transformed into AD494(DE3) cells. The recombinant clones were analyzed by restriction digestions, and positive clones were sequenced with an automated DNA sequencer.

Expression of the recombinant proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using anti-His antibodies. The recombinant proteins expressed in different prokaryotic expression vectors were analyzed by 10% SDS-PAGE for their subcellular localization. All the expressed recombinant proteins were purified on Ni^{2+}-NTA columns under denaturing conditions. Refolding conditions for each recombinant protein were standardized on a 16-well cell culture plate. Table 1 lists the refolding conditions used for the Ni^{2+}-NTA-purified F1.4 construct. Similar conditions were tried for all the recombinant Ni^{2+}-NTA purified proteins. The purified and refolded falcipain-1 proteins were analyzed for enzymatic activity by using a fluorogenic substrate, Z-F-R-AMC. Based on the yield and enzymatic activity, the recombinant protein F1.4 that contained the mature region and a 35-amino-acid pro domain of falcipain-1 was used for large-scale purification and characterization.

Large-scale expression and refolding of F1.4. Large-scale expression of F1.4 was carried out as follows. Bacteria transformed using pQE30-F1.4 plasmid were grown in 6 liters of culture medium to mid-log phase at 37°C. Cultures were induced with IPTG (1 mM) and allowed to grow further for 4 h at 37°C. Cells were harvested (~21 g [wet weight]), washed with ice-cold 100 mM Tris-HCl and 10 mM EDTA (pH 7.4), further disrupted by sonication for 30 cycles with 10-second pulsing and 10-second cooling intervals, and centrifuged at 12,000 × *g* for 30 min at 4°C. The pellet obtained was washed twice with 2 M urea, 20 mM Tris-HCl, 2.5% and Triton X-100 (pH 8.0), centrifuged at 17,000 × *g* for 30 min at 4°C, and solubilized in buffer A (6 M Gu-HCl, 20 mM Tris-HCl, 250 mM NaCl, 20 mM imidazole, pH 8.0) at room temperature (RT) for 60 min with gentle shaking on the rotator. Insoluble material was separated by centrifugation at 20,000 × *g* for 30 min at 4°C. The supernatant was incubated for 60 min at RT with Ni^{2+}-NTA resin preequilibrated with buffer A. The protein-bound resin was loaded onto a column and washed with 10 bed volumes each of (i) 6 M Gu-HCl, 20 mM Tris-HCl, 250 mM NaCl (pH 8.0), 8 M urea, 20 mM Tris-HCl, and 500 mM NaCl (pH 8.0) and (ii) 8 M urea, 20 mM Tris-HCl, and 30 mM imidazole (pH 8.0). Bound proteins were eluted with 8 M urea, 20 mM Tris-HCl, and 1 M imidazole (pH 8.0). The eluted proteins were analyzed by 10% SDS-PAGE and quantified by the Bradford dye-binding assay.

Thirty millligrams of Ni^{2+}-NTA-purified F1.4 was reduced with 10 mM DTT, and refolding was carried out in an optimized refolding buffer (100 mM Tris-HCl, 1 mM EDTA, 250 mM L-arginine, 2.5 mM reduced L-glutathione [GSH], 0.25 mM oxidized L-glutathione [GSSG] and 20% glycerol, pH 8.0) with a 100-fold dilution of ice-cold reduced denatured falcipain-1 to a final concentration of 20 μg/ml. Refolding was allowed to proceed at 10°C for 36 h with stirring, and the protein was concentrated using a tangential fast-flow concentrating system (Mil-

TABLE 1. Efficiency of refolding of F1.4 in a redox couple

GSH concn: GSSG concn (mM)	% Activity
10:1	22.3
7.5:1	15.5
5:1	8.2
2.5:1	0
1:1	0
10:0.5	17.1
7.5:0.5	20.8
5:0.5	30.0
2.5:0.5	15.1
1:0.5	4.3
10:0.25	0
7.5:0.25	0
5:0.25	6.8
2.5:0.25	100
1:0.25	9.4
0.5:0.25	7.6

lipore) to a final volume of 10 ml. An aliquot of the refolded falcipain-1 was analyzed by 10% SDS PAGE.

Preparation of recombinant falcipain-2 protein. Recombinant falcipain-2 was prepared as described previously by Shenai et al. (37) and Kumar et al. (14).

Enzyme activity of recombinant F1.4. Substrate gel analysis and fluorometric assay were performed to determine the enzyme activity of recombinant falcipain-1. Substrate gel analysis was performed as previously described (29) using SDS-PAGE under nonreducing conditions with gel copolymerized with 0.1% (wt/vol) gelatin. Recombinant protein samples were mixed with the nonreducing SDS-PAGE sample buffer and subjected to 10% SDS-PAGE under nonreducing conditions. Subsequently, the gel was washed twice with 2.5% Triton X-100 for 30 min each. The washed gel was incubated overnight in activity buffer (100 mM sodium acetate [pH.5.5], 10 mM DTT) and then stained with Coomassie brilliant blue and destained as per the standard protocol.

Fluorometeric assays for the recombinant refolded proteins were carried out in 3 ml assay buffer (100 mM sodium acetate [pH 5.5], 10 mM DTT) containing 200 nM enzyme. The fluorogenic substrate Z-F-R-AMC was added at 50 μM, and activity was monitored as the increase of fluorescence (excitation, 355 nm; emission, 460 nm) for 30 min at RT with an LS50B Perkin-Elmer fluorometer. For all assays the substrate concentration was saturating within the time courses of studies, such that curves for fluorescence measured over time remained linear. For inhibition studies, recombinant falcipain-1 was incubated with inhibitor for 10 min prior to the addition of substrate.

Characterization of F1.4 by PAGE and by gel permeation chromatography and mobility shift analysis. Refolded and purified falcipain-1 (F1.4) was analyzed by gel permeation chromatography using a Sephadex G-75 column (Amersham Pharmacia) on an AKTA fast protein liquid chromatography system (Amersham Pharmacia). The column was equilibrated with 20 mM Tris and 150 mM NaCl, pH 7.5. Standard peaks were acquired by running molecular weight protein markers. The void volume was determined by using blue dextran. Refolded protein was injected through a 500-μl loop, and the flow rate was maintained at 100 μl/min.

Refolded F1.4 (4 to 10 μg in 10 to 20 μl) was incubated with and without DTT at a final concentration of 100 mM at 37°C for 30 min. After this incubation, equal volumes of nonreducing SDS-PAGE sample buffer were added and both samples were boiled for 10 min. The samples were resolved by 10% SDS-PAGE and visualized by Coomassie blue staining.

Immunization of mice and rabbits. Two groups of six BALB/c mice (4 to 6 weeks old) were immunized intramuscularly with 50 μg of refolded F1.4 and falcipain-2, respectively, with proteins formulated in CFA. A control group of mice received only the adjuvant, emulsified in phosphate-buffered saline (PBS). Boosters were given on days 21 and 42 postimmunization with IFA. Mice were bled prior to immunization to collect preimmune sera. Blood samples were collected on days 0, 14, 35, and 56 postimmunization, and sera were analyzed by enzyme-linked immunosorbent assay (ELISA).

For rabbit immunization, two New Zealand White rabbits were immunized with 150 μg of recombinant falcipain-1 emulsified with CFA. Two booster immunizations were carried out on days 28 and 56 with protein formulated in IFA. Rabbits were bled on days 0 (preimmune), 42, and 70, and sera were analyzed by ELISA. Prior clearance for ethical animal experimentation was obtained for the in vivo experiments.

ELISA. ELISAs were carried out to evaluate the antibody responses generated against F1.4 in mice and rabbits. Briefly, 96-well microplates (Dynatech) were coated with 100 ng of F1.4 protein in 0.06 M carbonate-bicarbonate buffer (pH 9.6). The plates were kept overnight at 4°C, and wells were blocked with 5% low-fat milk in PBS (pH 7.2) for 1 h at room temperature. The antigen-coated wells were sequentially incubated with the appropriate dilutions of the respective antisera and optimally diluted with enzyme-labeled secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG). In between these incubations, plates were washed three times with 0.05% Tween 20 in PBS, followed by three washes with PBS only. The enzyme reaction was developed with *o*-phenylenediamine dihydrochloride-H_2O_2 in citrate phosphate buffer (pH 5.0) and stopped with 8 N H_2SO_4, and the optical density (OD) and 490 nm was measured using an ELISA microplate reader (Molecular Devices). On the basis of preimmune sera giving an OD of 0.07 ± 0.014 at a dilution of 1:200, an OD cutoff of 0.1 (mean ± 2 standard deviations) was selected for antibody titer determination.

Immunofluorescence assay. Antiserum to recombinant F1.4 was also tested in an immunofluorescence assay for reactivity with falcipain-1 and yoelipain-1 in the respective parasites. The assay was performed essentially as described earlier (31, 44). Briefly, multispot parasite slides were made from *P. falciparum* culture and from blood smears of *P. yoelii*-infected mice. The slides were air dried and fixed with acetone-methanol (9:1) at RT for 40 min. Polyclonal antibodies raised against F1.4 protein were diluted (1:500) with 0.5% bovine serum albumin in PBS–0.05% Tween 20, and the slides were incubated with the diluted antibodies in a sealed, moist box for 2 h at RT. The slides were washed with PBS–0.05% Tween 20 and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) at a 1:200 dilution in 0.5% bovine serum albumin in PBS for 1 h at room temperature in the dark. The slides were rewashed, air dried, and mounted with an antifade solution to retard photobleaching (Bio-Rad). Fluorescence was examined by use of a Nikon SE300 microscope with a 100× oil immersion objective.

Immunoblot analysis. To prepare *P. yoelii* parasite lysate, BALB/c mice were injected with 10^7 to 10^8 erythrocytes infected with *P. yoelii nigeriensis*. The course of infection was followed by making thin smears from the tail blood of the mice on alternate days. Infected mouse blood (~50% parasitemia) was drawn, mixed with heparin, washed by centrifugation three times in PBS at 700 × *g*, and passed through a CF 11 column to minimize the leukocyte contents (13). The eluted parasitized erythrocytes were then lysed with saponin, washed with PBS, and solubilized in SDS-PAGE buffer. *P. yoelii* lysate was subjected to 12% SDS-PAGE, and immunoblotting was performed with nitrocellulose membranes (Amersham Biosciences) blocked using 5% nonfat dry milk and 0.05% Tween 20 in PBS, pH 7.4. Blots were probed with anti-F1.4 antibody in phosphate-buffered saline (pH 7.4) containing 0.5% milk and 0.05% Tween 20. The secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (Sigma); reactions were detected using a West Pico Super Signal Enhanced Luminescence kit (Pierce).

Parasite challenge. The protective efficacy of F1.4 was studied by heterologous parasite challenge to immunized mice with sporozoites from the lethal mouse malaria strain *P. yoelii* ND67. Briefly, mosquitoes were infected with *P. yoelii* ND67 parasites, and thoracic regions were dissected out. The sporozoites were prepared by homogenizing the thoracic regions in normal saline containing 2% bovine serum albumin. Animals were challenged by intravenous inoculation of 10^4 sporozoites at 8 days after the last booster (day 50). Parasitemia was examined in Giemsa-stained thin and thick blood smears daily starting 4 days post-challenge.

RESULTS

Cloning, expression, and purification of different falcipain-1 constructs. Five different falcipain-1 constructs (F1.1, F1.2, F1.3, F1.4, and F1.5) were designed (Fig. 1). While the first four constructs differed from each other with respect to the length of the pro domain, the fifth construct (F1.5) consisted of 28 amino acids of the pro region of falcipain-2 and the full mature region of falcipain-1. Four falcipain-1 fragments (F1.2, F1.3, F1.4, and F1.5) were expressed in a number of *E. coli* expression systems. Moderate to high-level expression was seen for F1.2 in the pET32b system as a thioredoxin fusion, for F1.3 in the pET22b vector, and for F1.4 and F1.5 in the pQE30 system (data not shown). We did not observe significant levels of expression for the full-length falcipain-1 (F1.1) (data not shown). All the recombinant falcipain-1 fragments were expressed in insoluble form. Purification of the recombinant proteins under denatured conditions on Ni^{2+}-NTA affinity columns yielded about 80 to 90% homogenous proteins (Fig. 2A). The maximum yield and purity (~5 mg/liter and ~90% purity) were obtained for F1.4 protein expressed in the pQE30 vector system. These purified proteins were then refolded by rapidly diluting the proteins to a final concentration of 20 μg/ml with buffers which varied with respect to the ratio of oxidized to reduced glutathione. The refolded proteins were concentrated, estimated, and analyzed for the protease activity using fluorometric and gelatin hydrolysis assays on SDS-PAGE. Among the four constructs (F1.2, F1.3, F1.4, and F1.5), F1.4 showed the best yield after refolding in terms of protein amount and enzyme activity. Table 1 shows the activities of F1.4 in 16 different refolding conditions. Based on these preliminary results, we decided to pursue large-scale expression and purification of the F1.4 construct in the pQE30 expression system.

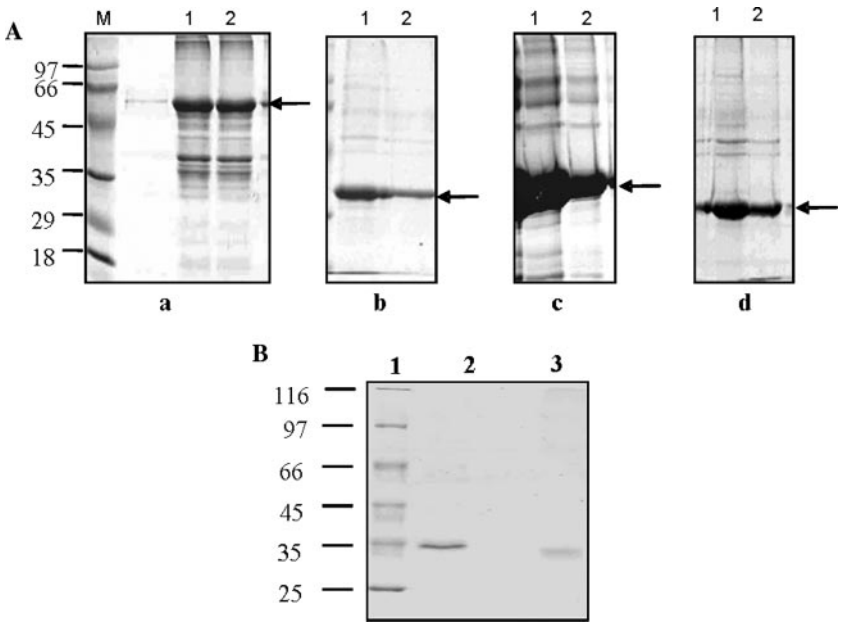

FIG. 2. (A) Coomassie blue-stained 10% SDS-polyacrylamide gel of Ni^{2+}-NTA-purified falcipain-1 fragments F1.2 (a), F1.3 (b), F1.4 (c), and F1.5 (d). Lane M, Molecular mass markers (kDa). Arrows indicate positions of the falcipain-1 protein fragments. Lanes 1 and 2, elutes from Ni^{2+}-NTA column. (B) Coomassie blue-stained SDS-polyacrylamide gel of purified and refolded F1.4. Lane 1, molecular mass markers (kDa); lane 2, reduced F1.4; lane 3, nonreduced F1.4.

Large-scale expression and refolding of F1.4. For the large-scale production of recombinant F1.4, culture was grown at a 6-liter scale. Ni^{2+}-NTA purification and refolding were carried out as described in Materials and Methods. During refolding a substantial amount of protein precipitated out; this could be due to the presence of aggregates of unfolded falcipain-1 protein. The soluble protein was concentrated using a tangential fast-flow concentrating system from Millipore, and part of it was dialyzed against buffer containing 100 mM sodium acetate and 20% glycerol (pH 5.5) for 10 to 15 h before activity analysis. After refolding, the yield of the recombinant protein was ~1 mg/liter.

Characterization of refolded F1.4. Refolded F1.4 was analyzed for its homogeneity by gel permeation chromatography. Purified recombinant F1.4 migrated as a single major peak (data not shown). F1.4 migrated as a ~31-kDa protein on SDS-PAGE and showed a mobility shift under reducing conditions compared to nonreducing conditions, thereby suggesting the formation of disulfide bonds (Fig. 2B). No dimers or higher-order oligomers were observed under nonreducing conditions on SDS-PAGE.

Activity analysis of F1.4. The enzyme activity of refolded F1.4 was analyzed using a fluorogenic substrate, Z-F-R-AMC. As shown in Fig. 3A, refolded F1.4 cleaved the fluorogenic substrate, and the proteolytic activity was higher at acidic pH than at neutral pH. The protease activity of the refolded protein was lost upon denaturation. E-64 at 10 μM and leupeptin at 100 μM completely inhibited the proteolytic activity of the enzyme. We failed to observe any hemoglobin degradation by F1.4.

We also assessed the activity of F1.4 by gelatin SDS-PAGE assay. A clearing was observed against the blue background at a molecular mass of ~32 kDa, suggesting a proteinase activity (Fig. 3B).

Production of anti-falcipain-1 antibodies. Immune responses against recombinant F1.4 formulated in CFA/IFA adjuvant in mice as well as in rabbits were determined by ELISA at different time points. F1.4-specific antibody was detected after the primary immunization, and antibody titers were boosted with each subsequent booster. In both mice and rabbits, the antibody response reached a peak after a second boost. The end point titers obtained ranged between 0.9×10^6 and 1.3×10^6 in mice, while two rabbits elicited titers of 2.8×10^6 and 3.0×10^6, respectively. The preimmune sera (day 0) or adjuvant control sera did not show reactivity with the recombinant protein in ELISA.

Detection of native falcipain-1 by anti-F1.4 antibodies. Antibodies to F1.4 raised in mice and rabbits tested positive in an immunofluorescence assay against blood stages of *P. falciparum*. Merozoites stained with an antibody to falcipain-1 showed a distinct staining at the apical end of the parasite next to the nucleus (Fig. 4A). A punctuate falcipain-1 staining pattern was also observed in erythrocytic schizonts (Fig. 4B). In addition, costaining was performed with antibodies to $MSP\text{-}1_{19}$ and the micronemal protein EBA-175. Merging of the two staining patterns revealed that compartments containing falcipain-1 are distinct from the MSP-1- and EBA-175-containing compartments (Fig. 4). The unique falcipain-1 location at the apical end of the merozoites suggested a role of falcipain-1 either in red blood cell (RBC) rupture or during reinvasion of host red blood cells.

Detection of yoelipain-1 by anti-F1.4 antibodies. To find out if anti-F1.4 antibody recognizes its ortholog yoelipain-1, we performed Western blotting with *P. yoelii* lysate. As shown in

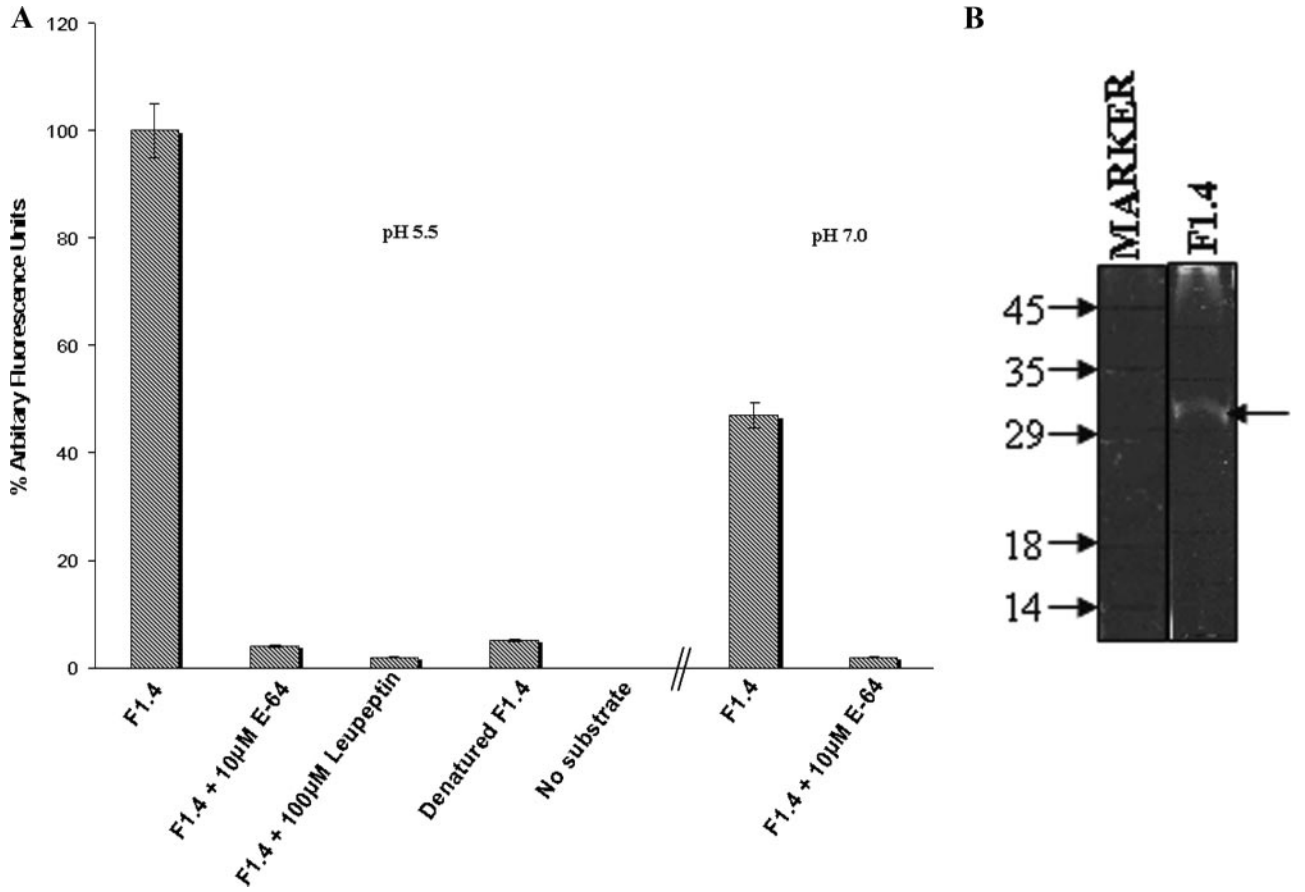

FIG. 3. (A) Proteolytic activity of F1.4 at pH 5.5 and pH 7.0. Activity was analyzed for the hydrolysis of Z-F-R-AMC and represented as the release of fluorescence in arbitrary fluorescence units measured over a period of 30 min (percentage of maximum activity). Error bars represent the standard deviations of the results from three independent refolding experiments. (B) Activity analysis of F1.4 on SDS-PAGE with gelatin as a substrate. Proteolysis is shown by a clear region on the gel as indicated by an arrow.

Fig. 5A, anti-F1.4 antiserum recognized a ~34-kDa band in *P. yoelii* lysate, while preimmune sera failed to recognize any band. These results were further confirmed by immunofluorescence assay against blood stage *P. yoelii* parasites; a punctuate staining was observed in *P. yoelii* schizonts (Fig. 5B). The results of Western blotting and immunostaining suggested that anti-F1.4 antibody significantly cross-reacts with the ortholog yoelipain-1.

Protective efficacy of falcipain-1 in mice. Since falcipain-1-specific inhibitors have been earlier shown to block merozoite reinvasion of RBCs (10), we further assessed the protective efficacy of falcipain-1 in a mouse model system. To do so, a group of six mice were immunized with recombinant F1.4 formulated in CFA and in IFA. The control group was immunized with adjuvant alone. Another group of six mice were immunized with recombinant falcipain-2, a trophozoite stage cysteine protease. Upon challenge with sporozoites (10^4) of the lethal strain *P. yoelii nigerensis* ND67, mice immunized with adjuvant alone developed a patent infection by day 4 to 5 postchallenge and reached a peak parasitemia of 85% ± 5% on day 9 ± 3 (Fig. 6A), while the mice immunized with falcipain-2 reached a peak parasitemia of 70% ± 5% on day 20 ± 3 ($P = 0.008$). In comparison, mice immunized with F1.4 became patent by day 6 to 7 postchallenge. This group showed slower growth of parasites and a significantly lower peak parasitemia of 10% ± 5% on day 22 ($P = 0.0025$). All the naïve and falcipain-2-immunized mice succumbed to infection by days 15 and 20, respectively, whereas mice vaccinated with F1.4 survived until day 30. The mice immunized with the recombinant F1.4 protein had a mean survival time of 30 ± 4 days, compared with 11 ± 4 days for the naïve mice (Fig. 6B). Death in F1.4-immunized mice can be attributed to reasons other than high parasitemia, as they maintained a fairly low parasitemia even before death.

DISCUSSION

Of the four falcipains observed in *P. falciparum* so far, falcipain-2A, -2B, and -3 have been biochemically and functionally well characterized and shown to be involved in degradation of hemoglobin and RBC membrane proteins (37, 40, 41). Although it was the first cysteine protease to be described, the role of falcipain-1 has remained unclear due to its relatively low abundance in erythrocytic stages of the parasite and difficulty in its expression using heterologous expression systems.

Attempts to produce falcipain-1 in baculovirus system (33) or as a fusion protein in an *E. coli* system have not been entirely satisfactory (9). Given the widespread experience with

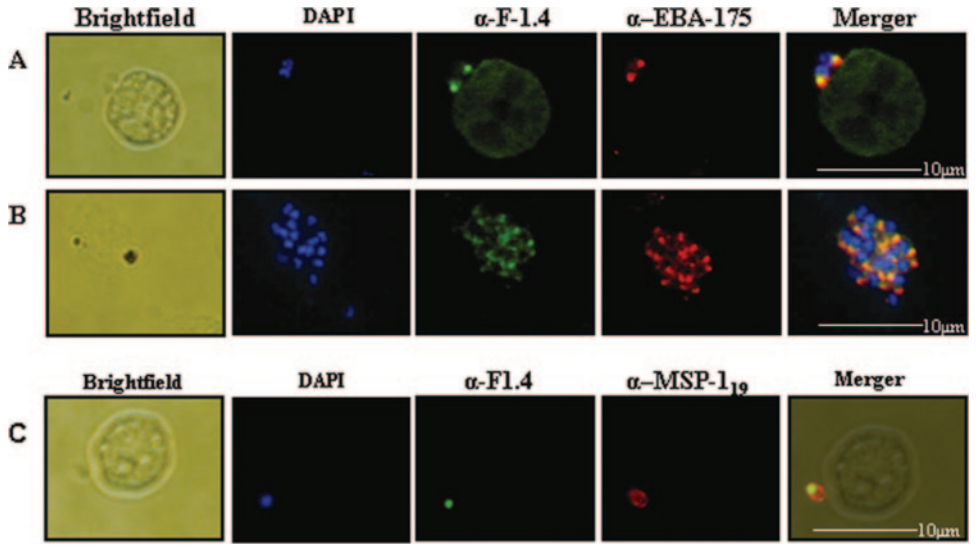

FIG. 4. Immunofluorescence images of fixed 3D7 *P. falciparum* parasites. (A) Merozoites costained with anti-F1.4 and anti-EBA-175 antibodies. (B) Schizonts costained with anti-F1.4 and anti-EBA-175 antibodies. (C) Merozoites costained with anti-F1.4 and anti-MSP-1_{19} antibodies.

and convenience of using *E. coli* as a heterologous system, we decided to carry out a systematic study with the objective of producing falcipain-1 in this system. However, most heterologous recombinant proteins expressed in *E. coli* are produced as insoluble proteins, and many are expressed as fusion proteins along with partners such as glutathione *S*-transferase or MBP, which, in addition to being useful in affinity purification, are believed to assist in the folding and solubilization of expressed proteins. However, these large fusion partners may interfere with the biological activity of the target proteins.

Considering that the expression of falcipain-1 in *E. coli* and other expression systems has been problematic (9, 33), we designed a number of constructs, all containing the mature catalytic region of falcipain-1 along with different lengths of the pro domain of the enzyme; pro domains are recognized to play a major role in the folding and stability of many proteases (37, 40, 41, 46). The construct F1.5 was a hybrid construct, designed in a way that it expresses a 28-residue-long peptide from the pro domain of the falcipain-2A sequence fused to the mature region of falcipain-1. This peptide sequence has been shown to play a crucial role in the folding process of falcipain-2 (37). Several attempts were made to express the five falcipain-1 constructs by using different *E. coli* expression vectors and a variety of *E. coli* strains. Protein expression varied from poor to reasonable levels. The pQE30 vector appeared to be more suitable for constructs F1.4 and F1.5, while pET vectors gave

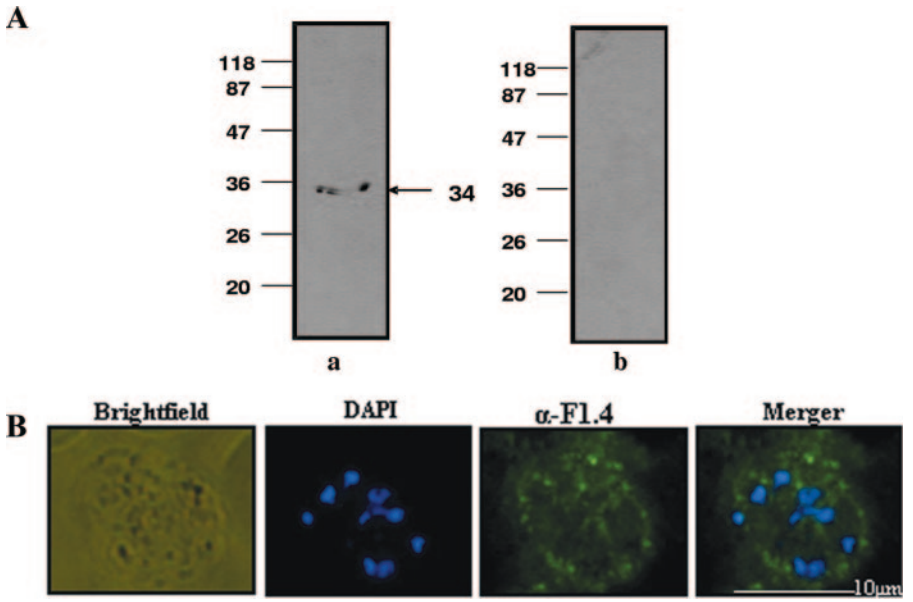

FIG. 5. (A) Immunoblot analysis of *P. yoelii* lysate using anti-F1.4 antiserum (a) and when probed with preimmune sera (b). (B) Immunofluorescence images of *P. yoelii* parasites immunostained with anti-F1.4 antibody.

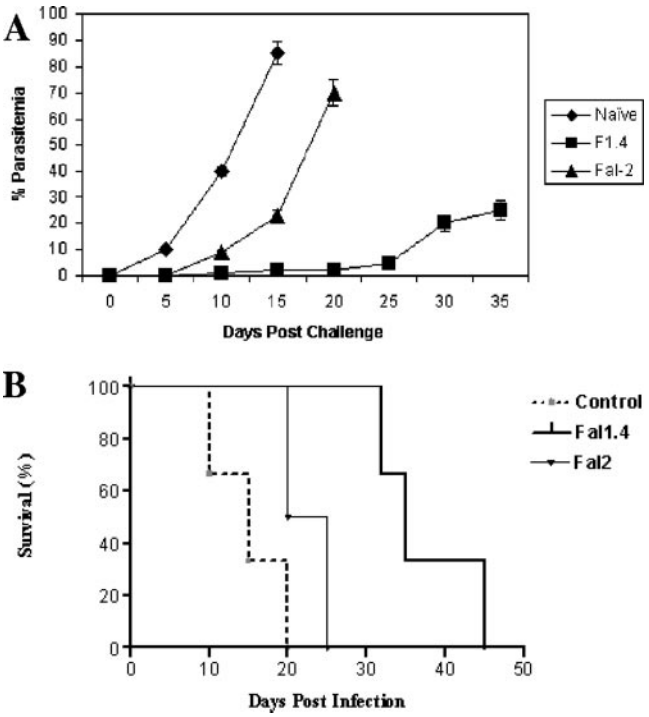

FIG. 6. (A) Parasitemia profiles of mice immunized with F1.4 and falcipain-2 protein when challenged with *P. yoelii* sporozoites. The percent parasitemia in mice was recorded for 35 days postchallenge. (B) Percent survival of mice immunized with F1.4 and falcipain-2 when challenged with *P. yoelii* sporozoites.

better results for F1.2 and F1.3. These results emphasize the unpredictable nature of heterologous expression systems and the need to try multiple expression systems/conditions (22).

All the recombinant falcipain-1 fragments were produced in insoluble forms in different *E. coli* expression systems. To obtain an active falcipain-1, proteins were solubilized in denaturing conditions, purified by Ni^{2+}-NTA chromatography, and refolded using different refolding conditions. The refolded falcipain-1 proteins showed different levels of activity and yields. The best activity and yield were obtained for F1.4 protein. The F1.4 protein migrated as a monomer on gel permeation chromatography and showed a mobility shift on SDS-PAGE in nonreducing conditions, thereby suggesting the formation of disulfide bonds in the recombinant protein.

The results of fluorometric assays using the peptide substrate Z-F-R-AMC showed that the protein is active at acidic pH and the activity is significantly reduced at neutral pH. The enzyme activity was completely abolished by cysteine protease inhibitors, E-64 and leupeptin. These observations are in contradiction with the findings of Goh et al. (9), who reported that a recombinant falcipain-1 preparation was more active at neutral pH than at acidic pH against the benzyloxycarbonyl-Leu-Arg-7-amino-4-methyl-coumarin fluorogenic substrate. Further, and more surprisingly, the most commonly used cysteine protease inhibitor, E-64, did not inhibit the protease activity of their preparation. Goh et al. expressed falcipain-1 as a fusion partner with MBP, which showed no protease activity, leading those workers to cleave off MBP with factor Xa protease to obtain the recombinant falcipain-1 for biochemical characterization. It is not clear whether the observed discrepancies are due to a difference in size or conformation of the recombinant falcipain-1 preparations or to the method of production of the protein. However, our results are in line with the earlier work done with falcipain-1 by Salas et al. (33), although we failed to observe any hemoglobin degradation by F1.4. A recent report identified a 10-amino-acid motif in the C-terminal region of falcipain-2 that binds hemoglobin (21). This motif is distant from the enzyme active site and protrudes out from the protein. However, the sequence of the corresponding motif in falcipain-1 varies considerably, and it remains to be seen whether this motif in falcipain-1 binds to hemoglobin.

Parasite cysteine proteases have been shown to be immunogenic and are being exploited as serodiagnostic markers and vaccine targets (15, 32). These cysteine proteases also seem to play key roles in immunoevasion (1); enzyme activation, virulence, and tissue and cellular invasion (18); excystment/encystment (47); and hatching and molting (16, 25). Anti-*Fasciola hepatica* cathepsin L antibodies, anti-*Dermatophagoides vivipparous* cysteine protease, and anti-*Dermatophagoides pteronyssinus* Der p1 antibodies are inhibitory to the proteolytic activity of these proteases (4, 43). Antibody-mediated protection has been reported with *P. falciparum* SERA/SERP H antigens in *Aotus* monkeys and mice (11, 45), with *Leishmania major* cysteine protease in mice (49), with *Entamoeba histolytica* cysteine protease antigens in vitro (23), and also with *Schistosoma mansoni* calpains (12). Possibilities for anti-cysteine protease vaccines against parasitic organisms has been reported for *Fasciola hepatica* and *Haemonchus contorius* infections (6, 42, 43), as well as for *Trypanosoma cruzi* (34, 35). Protection of mice against gastric colonization by *Helicobacter pylori* by immunization with urease subunits A and B has also demonstrated vaccine potential of proteases (5). These reports and recent suggestions that falcipain-1 is involved in the merozoite invasion of erythrocytes led us to explore the immunogenicity of F1.4 in small animals.

Both mice and rabbits immunized with recombinant refolded falcipain-1 (F1.4) elicited high humoral responses, indicating the presence of both B- and T-cell epitopes in recombinant F1.4. We used these anti-F1.4 antibodies for immunolocalization and for invasion inhibition of erythrocytes by the malaria parasite. The results show that falcipain-1 was localized in discrete compartments in the schizont and merozoite stages, and anti-falcipain-1 antibodies inhibited merozoite invasion by 30% (data not shown). We also studied the protective efficacy of falcipain-1 in a mouse model against a heterologous *P. yoelii* challenge. This model was chosen because the yoelipain-1 enzyme shows ~55% sequence identity to falcipain-1 (28). A characteristic infection was observed in naïve mice, whereas immunized mice showed a delay in the onset of infection and a significant reduction in the total parasite burden. Importantly, immunized mice died much later than the control mice. It seems that reduction in parasitemia observed in F1.4-immunized mice is not sufficient to protect mice from death following a challenge infection with a lethal strain of *P. yoelii*. Similar results have been reported for mice immunized with malaria vaccine candidate antigens and challenged with *P. yoelii* (30). These results suggest that falcipain-1 may have a role in the asexual blood stages of the malaria parasite. A recent study using a falcipain-1-specific inhibitor (YA29) has also suggested

a role for falcipain-1 in merozoite invasion (10). In contrast, two more recent studies have shown that falcipain-1 knockout parasites develop normally, indicating that falcipain-1 is not essential for RBC invasion (7, 38). However, it is possible that the loss of falcipain-1 function in the knockout parasite line is compensated by other cysteine proteases or an alternate invasion pathway that becomes operational when knockout parasites are selected under drug pressure, as seen in case of the EBA-175 knockout parasite line (24). It is also possible that while falcipain-1 might not be absolutely essential for *P. falciparum*, yoelipain-1 may yet have an essential role in the intraerythrocytic cycle of *P. yoelii*, as indicated by the present study. We were also able to use falcipain-2A immunization as a separate control for our challenge experiment and found that these immunized mice succumbed to infection much earlier than the F1.4-immunized mice.

In conclusion, we show here that falcipain-1 can be expressed in an *E. coli* expression vector without any bulky fusion partner. The refolded protein exists in monomeric form, is enzymatically active, and can be used for structural studies. The recombinant falcipain-1 is highly immunogenic and generates a protective immune response. These results suggest that falcipain-1 may be further developed as a component of a multistage, multisubunit vaccine.

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