Ankyrin Peptide Blocks Falcipain-2-mediated Malaria Parasite Release from Red Blood Cells

Falcipain-2 (FP-2) is a dual-function protease that cleaves hemoglobin at the early trophozoite stage and erythrocyte membrane ankyrin and protein 4.1 at the late stages of parasite development. FP-2-mediated cleavage of ankyrin and protein 4.1 is postulated to cause membrane instability facilitating parasite release in vivo. To test this hypothesis, here we have determined the precise peptide sequence at the hydrolysis site of ankyrin to develop specific inhibitor(s) of FP-2. Mass spectrometric analysis of the hydrolysis products showed that FP-2-mediated cleavage of ankyrin occurred immediately after arginine 1,210. A 10-mer peptide (ankyrin peptide, AnkP) containing the cleavage site completely inhibited the FP-2 enzyme activity in vitro and abolished all of the known functions of FP-2. To determine the effect of this peptide on the growth and development of P. falciparum, the peptide was delivered into intact parasite-infected red blood cells (RBCs) via the Antennapedia homeoprotein internalization domain. Growth and maturation of trophozoites and schizonts was markedly inhibited in the presence of the fused AnkP peptide. <10% of new ring-stage parasites were detected compared with the control sample. Together, our results identify a specific peptide derived from the spectrin-binding domain of ankyrin that blocks late-stage malaria parasite development in RBCs. Confocal microscopy with FP-2-specific antibodies demonstrated the proximity of the enzyme in apposition with the RBC membrane, further corroborating the proposed function of FP-2 in the cleavage of RBC skeletal proteins.

Plasmodium falciparum causes the most severe form of human malaria and is responsible for nearly all malaria-induced mortality. Clinical manifestations of malaria are caused by the intraerythrocytic life cycle of P. falciparum. The parasite undergoes distinct morphologic changes during its 48-h life cycle inside human red blood cells (RBCs) (1). After invasion into RBCs, the parasites mature from ring stage to trophozoites and then to schizonts. Mature segmented schizonts finally rupture the host erythrocytes releasing merozoites, which rapidly invade other erythrocytes to reinitiate the cycle.

The mechanism of merozoite release from host RBCs is largely unknown. The cluster of merozoites inside a red blood cell is enclosed within two membranes: an inner parasitophorous vacuole membrane (PVM) and an outer RBC membrane. The rupture of these two membranes precedes the release of merozoites for subsequent round of RBC invasion. Several studies have shown that the merozoite release is susceptible to protease inhibitors. In the presence of such inhibitors, merozoites mature normally but are unable to escape from RBCs (2–4). Furthermore, a number of plasmodial proteases have been isolated and are shown to have activities against known RBC membrane skeletal proteins (5), although a functional role of these proteases in the rupture of the RBC membrane during merozoite escape has not been established.

The issue of malaria parasite release from RBCs was recently examined by two separate research groups. By using a combination of E64, a cysteine protease inhibitor, and a specific antibody labeling approach, Salmon et al. (6) proposed a two-step process for parasite release that includes an initial exit of merozoites enclosed within the PVM followed by a rapid parasite escape by a proteolysis-dependent mechanism. Secondly, Winograd et al. (7) used video microscopy to study the release of merozoites and concluded that an apertural is made through the PVM as well as the red cell membrane to allow merozoite exit in an orderly fashion. Another report from the same group (8) detected fragments of erythrocyte membrane and PVM in culture medium at the time of merozoite release from infected RBCs. This study implied that although there might be two distinct components underlying the release mechanism, they operate together to ensure a simultaneous breakdown of the two barriers encapsulating the intracellular parasite.

The present evidence as outlined above suggests that proteases are probably involved in the parasite release, but the precise molecular mechanism underlying this process remains largely uncharacterized. In this context, parasite-derived proteases that are involved in the breakdown of certain host cytoskeletal proteins at different stages of parasite development are of interest as potential therapeutic targets. Previously, we showed that P. falciparum derived cysteine protease falcipain-2 (FP-2) cleaves host erythrocyte membrane skeletal proteins at neutral pH. Specifically, FP-2 cleaves ankyrin and protein 4.1, the cytoskeletal elements vital to the stability of RBC membrane (9, 10). This proteolytic event is accompanied by membrane instability as evident by an increased rate of

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‡To whom correspondence should be addressed: Division of Hematology Research, ACH 406, St. Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135. Tel.: 617-789-2677; Fax: 617-789-3111; E-mail: manjit.hanspal@tufts.edu.

The abbreviations used are: RBC, red blood cell; FP-2, falcipain-2; PVM, parasitophorous vacuolar membrane; Ant, antenapedia protein; PBS, phosphate-buffered saline; Ni-NTA, nickel-nitrilotriacetic acid; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; IOVs, inside-out vesicles; rFP-2, recombinant FP-2; SERP, serine-rich protein; AnkP, ankyrin peptide.
membrane fragmentation of infected RBCs. We also documented that FP-2 cleaves protein 4.1 within a region of the spectrin-actin binding domain critical for erythrocyte membrane stability and identified a 16-mer peptide that inhibits FP-2 in vitro with a $K_i$ of 100 μM (10). In our continuing pursuit to develop potent inhibitors of falcipain-2, in the present study, we have identified the precise peptide sequence at the hydrolysis site of ankyrin. We also demonstrate that a 10-mer ankyrin peptide containing the cleavage site inhibits FP-2 in vitro with a $K_i$ of 2 μM and effectively blocks late-stage malaria parasite development in RBCs. These studies support our previous hypothesis and provide an important framework for the development of peptide-based antimalarial drugs.

EXPERIMENTAL PROCEDURES

Parasite Culture and Preparation of Parasite Extract—P. falciparum (strain 3D7) culture was maintained in vitro in the presence of 15% human serum in a culture medium containing fresh A/H11001 NaHCO₃, L-glutamine, and pyruvic acid as described previously (11). Parasites were grown up to 6% parasitemia in the presence of 15% sorbitol-Percoll (Sigma) gradients (13). For the preparation of parasite extracts, RBCs infected with trophozoites were incubated with 0.01% (w/v) saponin in PBS at 37°C for 10 min to lyse erythrocyte membrane followed by three washings with ice-cold PBS. Released parasites were then lysed with 5 mM phosphate buffer, pH 8.0, and centrifuged at 100,000 × g for 30 min (14). The resulting supernatant is referred to as “soluble parasite extract.”

Expression of Recombinant FP-2 and FP-3—FP-2 encoding the complete mature domain plus 35 amino acids (from the COOH-terminal end) of the prodomain was expressed in M15(pREP4)-strain Escherichia coli and refolded to obtain mature active enzyme as described previously (15). An identical protocol was used to obtain recombinant FP-3, which encoded the complete mature domain plus 33 amino acids (from the COOH-terminal end) of the prodomain (16). In this study, recombinant FP-3 was used only for testing the specificity of FP-2 antibodies.

Construction and Expression of His-tagged Recombinant Ankyrin—To identify the precise cleavage site, we designed a cDNA construct containing nucleotides 3001–4560 encoding amino acids 972–1,491 (17). The insert was PCR-amplified from a human reticulocyte cDNA library and ligated into the pQE-30 vector, which encodes an NH₂-terminal His tag (Qiagen) to generate an expression construct. After confirming the sequence, the cDNA construct was transfected into E. coli and the transformants were induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside and analyzed by SDS-PAGE for the expression of the fusion protein termed (His)₆-ank972.

Recombinant His-tagged ankyrin segment was bound to Ni-NTA resin and digested with 0.2 μM FP-2 at pH 7.5 for 30 min at 37°C. Bound protein was then eluted with 8 M urea, 20 mM Tris-HCl, 1 M imidazole, pH 8.0, and subjected to MALDI-MS at Harvard Microchemistry Laboratory (Cambridge, MA) to determine the precise molecular mass of the cleaved product. To further examine whether additional cleavage sites were located in the COOH-terminal half of the recombinant protein, the 972–1,491 amino acid segment of ankyrin was cloned into pET22a vector (Novagen), which encodes His tag at both ends. The NH₂-terminal tag was removed by thrombin cleavage (according to the manufacturer’s instructions). Resin was washed thoroughly and digested with FP-2 as described above. The sample was centrifuged, and both the supernatant and pelletted beads were analyzed by SDS-PAGE.

Measurement of Protease Activity—To detect enzyme activity against erythrocyte membrane, intact E. coli and the transformants were incubated with isopropyl-1-thio-β-D-galactopyranoside and analyzed by SDS-PAGE. To detect the enzyme activity against hemoglobin, 100 nM rFP-2 was added into 25-μl reaction mixture containing 3 μg of human IgG Fc (Sigma) in 100 mM sodium acetate, pH 5.5, 1 mM diithiothreitol. Reactions were carried out at 37°C for 60 min, and digestion products were analyzed by 15% SDS-PAGE.

Generation of Peptide-specific Antibodies—A peptide corresponding to residues 185–197 (EIVYNPLTKKGKEKH) of FP-2 with an additional cysteine residue at the COOH terminus was synthesized and linked to keyhole limpet hemocyanin. Antibodies to the conjugate were raised in rabbits. Peptide synthesis and antibody production was performed by Cell Essentials (Boston, MA). The antisera was tested for immunospecificity for FP-2 by Western blots of P. falciparum parasite extract. Peptide-specific antibodies were obtained by affinity purification of antisera on cyogen bromide-immobilized peptide. Antibodies were eluted with 0.1 M glycine, pH 2.5, immediately neutralized with Tris and dialyzed against phosphate-buffered saline.

Immunofluorescence—Parasite culture enriched in trophozoites was washed three times in PBS, and cells were smeared on glass slides, fixed in ice-cold methanol for 20 min, air-dried, and blocked in 1% nonfat milk in PBS for 30 min at room temperature. Slides were then incubated for 90 min at room temperature in primary antibodies diluted in blocking buffer (1:60 for the affinity-purified FP-2 antibody and 1:30 for serine-rich protein (SERP), plasmapepsin IV, or LWL1 antibodies). SERP antibodies were generously provided by Dr. Stefan Baumeister (Marburg, Germany); plasmapepsin IV was provided by Dr. Daniel Goldberg (Washington University School of Medicine, St. Louis, MO); and LWL1 antibodies were obtained from the MR4 program (MRA-73, a gift from Dr. Kasturi Halder, Stanford University, Stanford, CA). Slides were then washed in PBS and incubated for 60 min at room temperature with secondary antibodies purchased from Molecular Probes (Alexa-488-conjugated (green) goat anti-rabbit IgG or Alexa 594-conjugated (red) goat anti-mouse IgG secondary antibody) diluted 1:100 in blocking buffer. Slides were washed in PBS, air dried, and mounted with Antifade (Molecular Probes), and coverslips were sealed. Slides were viewed using Leica TCS SP2 confocal fluorescence microscope, and images were analyzed and prepared for publication using Adobe Photoshop.

Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-PAGE was performed essentially by the method of Laemmlı (18). Western blotting was performed essentially as described by Towbin et al. (19). The immune reactive bands were detected using an enhanced chemiluminesence (ECL) system (Amersham Biosciences).

RESULTS

Falcipain-2 Cleaves Erythrocyte Ankyrin within the Spectrin-binding Domain—In our initial attempt to identify the cleavage site of ankyrin, we used two previously defined monoclonal antibodies, 8C3 and 2H1, recognizing epitopes in different regions of the molecule. The epitope for 8C3 lies somewhere between residues 1 and 1,012, and that for 2H1 lies within residues 1,462 and 1,798 in the regulatory domain of human erythrocyte ankyrin (14). We showed that the 155-kDa truncated ankyrin cross-reacted with 8C3 antibody but not with the 2H1 monoclonal, suggesting that it lacks most of the COOH-terminal regulatory domain (14). This result, together with the size of the cleaved product, suggests that FP-2 cleaves erythrocyte ankyrin at a site between the COOH-terminal half of spectrin-binding domain and the beginning of the regulatory domain.

To identify the precise cleavage site, we designed a construct to express a fragment of ankyrin from amino acids 972 to 1,491 that covers most of the spectrin-binding domain and a small portion of the regulatory domain. This fragment was expressed in E. coli as a (His)₆-ank972 fusion protein, which migrated on SDS-PAGE with an apparent molecular mass of ~60 kDa (Fig. 1B, lane 1). Recombinant protein was bound to Ni-NTA resin and digested with 0.2 μM FP-2 at pH 7.5 for 30 min at 37°C. After incubation, the sample was centrifuged and both the supernatant and pelletted beads were analyzed by SDS-PAGE.

The supernatant and the pelletted beads were analyzed by SDS-PAGE. Although no protein was detected in the supernatant (data not shown), a major protein band with an apparent molecular mass of ~27 kDa was detected in the pellet (Fig. 1B, asterisk). In addition, a band corresponding to undigested recombinant protein was detected. Immunoblot analysis confirmed that the ~27-kDa band was a fragment of ankyrin and it contained the NH₂-terminal His tag, suggesting that the cleavage took place from the COOH-terminal end of the recombinant protein. Bound protein was then eluted with 8 M urea,
Nickel-bound protein eluted in minimum volume of a buffer containing 1 M imidazole. This sample was subjected to MALDI-MS analysis.

Arrowhead vector, which encodes His6 tag at both ends (Fig. 2). Recombinant ankyrin from amino acids 972 to 1,491 was cloned into pET32a vector, which encodes 109 amino acid thioredoxin protein (Trx Tag) and a thrombin cleavage site (indicated by arrow) at the amino terminus and a His6 tag at each end. B, SDS-PAGE analysis. Lane 1, recombinant protein bound to Ni-NTA beads; lane 2, nickel-bound ankyrin digested with 0.2 μM rFP-2 at pH 7.5 for 30 min at 37 °C and centrifuged to collect beads. A prominent protein band (indicated by an asterisk) containing the His tag was detected. Lane 3, nickel-bound protein eluted in minimum volume of a buffer containing 1 M imidazole. This sample was subjected to MALDI-MS analysis. Arrowhead indicates the position of the predicted FP-2 cleavage site.

Fig. 1. FP-2-mediated cleavage of ankyrin. A, The structure of human erythrocyte ankyrin containing three structural domains. A segment of human erythrocyte ankyrin from amino acids 972 to 1,491 was expressed in bacteria with a His6 tag at the NH2 terminus. B, SDS-PAGE analysis. Lane 1, recombinant ankyrin bound to Ni-NTA beads; lane 2, nickel-bound ankyrin digested with 0.2 μM rFP-2 at pH 7.5 for 30 min at 37 °C and centrifuged to collect beads. A prominent protein band (indicated by an asterisk) containing the His tag was detected. Lane 3, nickel-bound protein eluted in minimum volume of a buffer containing 1 M imidazole. This sample was subjected to MALDI-MS analysis. Arrowhead indicates the position of the predicted FP-2 cleavage site.

Fig. 2. FP-2 cleaves ankyrin at arginine 1,210. A, human erythrocyte ankyrin segment encoding amino acids 972–1,491 was cloned into pET32a vector, which encodes 109 amino acid thioredoxin protein (Trx Tag) and a thrombin cleavage site (indicated by arrow) at the amino terminus and a His6 tag at each end. B, SDS-PAGE analysis. Lane 1, recombinant protein bound to Ni-NTA beads; lane 2, Ni-NTA beads digested with thrombin. After thrombin digestion, beads were washed, digested with FP-2, and centrifuged to separate pellet (lane 3) and supernatant (lane 4). Arrowhead indicates the position of the predicted FP-2 cleavage site.

The effect of synthetic peptides was also tested on FP-2-mediated cleavage of ankyrin, rFP-2 was incubated with recombinant ankyrin fragment bound to Ni-NTA resin in the presence of increasing amounts of peptides (dissolved in Me3SO). Control samples contained the same concentration of Me3SO. As shown in Fig. 3A, peptide AnkP inhibited FP-2-mediated cleavage of ankyrin in a dose-dependent manner. Quantification of the intensity of 27,600-Da band (indicated by an asterisk) by densitometry showed that the peptide AnkP did not inhibit FP-2 activity between 0.1 and 1.0 μM (lanes 3 and 4) but caused nearly 100% inhibition at concentrations of 5.0 μM and above (lanes 5–7) with an inhibition constant Ki of ~2 μM. In contrast, the AnkPR peptide had no significant effect on FP-2 activity as compared with the control sample.
mediated cleavage of intact ankyrin and protein 4.1 associated with erythrocyte membrane IOVs. rFP-2 was incubated with IOVs at pH 7.5 in the presence of various amounts of each peptide, and the digestion products were analyzed by SDS-PAGE. As reported earlier (9, 10), FP-2-mediated cleavage of ankyrin and protein 4.1 produces a 155- and a 56-kDa protein fragment, respectively. Monitoring the intensities of these protein bands showed that peptide AnkP inhibited FP-2-mediated cleavage of both ankyrin and protein 4.1 in a dose-dependent manner, whereas the AnkPR peptide had no such effect. We also examined the effect of these peptides on FP-2-mediated hemoglobin degradation as described earlier (10). At concentrations of 5.0 μM and above, the AnkP peptide caused nearly 100% inhibition of hemoglobin degradation, whereas the AnkPR peptide had no effect at similar concentrations (data not shown).

We next investigated the effect of ankyrin peptides on parasite growth in vitro. Peptides were synthesized by fusing with the 16 amino acid internalization sequence of Antennapedia (Ant) homeoprotein, which permits the uptake of peptides into cells via facilitated diffusion (21, 22). Human erythrocytes infected with stage-specific P. falciparum were cultured in the presence of Ant-AnkP peptide, the Ant peptide alone, AnkP peptide alone, or a mutant form of the Ant-AnkP peptide where the AnkP portion was scrambled to eliminate the signature sequence of the cleavage site (Table I). As shown in Fig. 4, when highly synchronized ring-stage parasites were incubated with either peptide for 24 h, parasites matured to trophozoites in all of the cases (top panel). However, when trophozoite-stage parasites were incubated under identical conditions, virtually no rings were detected in cultures containing the Ant-AnkP peptide. The parasites appeared as condensed round structures, which failed to develop into segmented schizonts or rings (middle panel). In contrast, in control cultures (i.e. those containing Ant peptide, Ank peptide, or the mutant peptide), nearly all of the parasites developed into rings. Quantification of these data is shown in Table II. In the presence of Me2SO, AnkP, or Ant, the trophozoites matured normally giving rise to nearly 100% ring-stage parasites, whereas in the presence of increasing amounts of Ant-AnkP, there was a significant decline in the number of rings and, as expected, the scrambled peptide had no effect on ring formation. Similarly, when the schizont-stage parasites were incubated overnight, virtually no new rings were detected in cultures containing the Ant-AnkP peptide, indicating almost complete block of cell rupture and merozoite release (Fig. 4, bottom panel). The internalization of the Ant-AnkP peptide but not that of Ant, Ank peptide, or the mutant peptide was confirmed by incubating parasite-infected erythrocytes with the biotin-tagged versions of the peptides for 30 min at 37°C followed by visualization of the peptides using streptavidin-fluorescein isothiocyanate (data not shown).

Localization of Falcipain-2 in Parasite-infected Red Blood Cells—Using biochemical and immunofluorescence assays, a previous study (15) has suggested that the active mature falcipain-2 is present largely in the food vacuole of trophozoites. This finding appears to be inconsistent with the proposed role of FP-2 in the cleavage of the erythrocyte membrane skeleton. To address this issue, we investigated the cellular localization of the FP-2 enzyme using an antibody generated against a unique COOH-terminal peptide sequence found in the mature FP-2 enzyme. Immunoblot analysis showed that the affinity-purified antibody recognized both the inactive proenzyme and the active mature form of recombinant FP-2 (Fig. 5, lanes 1 and 2, respectively). This antibody also detected native FP-2 in the trophozoite extract (Fig. 5, lane 3), which apparently contains largely the mature active FP-2. The specificity of this antibody was further confirmed using recombinant FP-3, which shares a high degree of sequence similarity with FP-2 (16). As shown in Fig. 5, lane 4, the FP-2 antibody did not cross-react with recombinant FP-3.

The FP-2 antibody was used to visualize the location of falcipain-2 in synchronized parasite cultures by confocal fluorescence microscopy. Smears of trophozoite-infected erythrocytes were fixed in methanol and examined using the affinity-purified falcipain-2 antibody. As shown in Fig. 6A, the antibody stained the entire trophozoite. In addition, fluorescently labeled vesicles or vesicle-like structures of unknown identity were seen extending into the erythrocyte cytoplasm. No stain-
tein was present in nitrocellulose membrane confirmed that an equivalent amount of pro-
bands were detected with an ECL system. Ponceau S staining of the 197 of FP-2. Immunoreactive –
peptide corresponding to residues 185 and probed with affinity-purified antibody raised against the synthetic
separated by SDS-PAGE, transferred to a nitrocellulose membrane,
1 lanes 2 and 4. The size of the markers in kDa is shown on the left.

Fig. 4. Effect of ankyrin peptide on the growth and development of parasites. P. falciparum (strain 3D7) was cultured in fresh A+ human erythrocytes according to the method of Trager and Jensen (11). Synchronized cultures of parasite infected RBCs were incubated with 250 μM Ant-AnkP peptide or the scrambled Ant-AnkP peptide (labeled as Control) in a total volume of 1.0 ml for 20–24 h. Stocks of peptides were made in Me_2SO and diluted in culture medium. All of the samples contained same final concentration of Me_SO (0.15% (v/v)). Thin smears were stained with Giemsa and photographed.

Trophozoite-infected human erythrocytes (5% parasitemia) were cultured in a total volume of 1.0 ml for 20-24 h in the presence of Me_SO (0.15%, v/v), AnkP peptide, Ant peptide, Ant-AnkP peptide, or the scrambled Anti-AnkP peptide. Giemsa-stained smears were prepared, and ring-infected RBCs were counted as a percentage of total RBCs. These experiments were performed three times with similar results.

| Treatment          | Concentration | Ring parasites |
|--------------------|---------------|---------------|
| Me_SO              | 250           | 100           |
| AnkP               | 250           | 100           |
| Ant                | 250           | 95            |
| Ant-AnkP           | 50            | 73            |
|                   | 100           | 40            |
|                   | 250           | <10           |
| Scrambled Ant-AnkP | 250           | 100           |

SERP staining was observed in a ring form surrounding the parasite (Fig. 6B), and the LWL1 antibody showed a punctate staining pattern consistent with the localization of LWL1 antigen in the intraerythrocytic space of the infected erythrocyte (Fig. 6D). Co-labeling experiments with FP-2 and SERP antibodies could not be done because both antibodies were of rabbit origin. A comparison of FP-2 and SERP staining shows that the FP-2 is localized in the PVM as well as in a compartment that is distinct from the PVM. This conclusion is further strengthened when images of FP-2 and LWL1-staining patterns were merged. FP-2 appears to go beyond the area of LWL1 staining. Together, these results suggest that the FP-2 enzyme is in apposition with the inner surface of the erythrocyte membrane.

DISCUSSION

The precise mechanism of merozoite release from host red blood cells is largely unknown; however, considerable evidence now suggests that proteases play an important role in this process. In the erythrocytic stage of malaria parasite, several proteases including cysteine, aspartic, and metalloproteases have been characterized. A number of such proteases are active in hydrolyzing the RBC hemoglobin and are thus probably not relevant to the merozoite release. However, other proteases are expressed at the late stages of parasite development and might play a role during merozoite release from RBCs. Previous studies have shown that the cysteine protease FP-2 is a dual function protease that cleaves hemoglobin at the early trophozoite stage and targets specific components of the erythrocyte membrane skeleton at the late stages of parasite development. We have hypothesized that this latter action of FP-2 may be relevant to the release of malaria parasite from RBCs.

Previously, we showed that falcipain-2 cleaves erythrocyte membrane ankyrin near the carboxyl terminus. The removal of the COOH terminus of ankyrin weakened its interaction with the erythrocyte membrane and destabilized the skeletal network. Based on these findings, we postulated that protease-induced ankyrin degradation destabilizes the erythrocyte membrane skeleton, which in turn facilitates parasite release (14). To test this hypothesis, we initiated studies to determine the precise peptide sequence at the hydrolysis site of ankyrin with the intent to develop specific inhibitor(s) that might block or inhibit parasite release in vivo. To identify the precise cleav-
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Fig. 6. Immunolocalization of FP-2 by confocal microscopy. Erythrocytes infected with *P. falciparum* trophozoites were smeared on a glass slide, fixed with ice-cold methanol, and incubated with polyclonal FP-2 (A) or SERP (B) antibodies followed by Alexa 488 (green) or Alexa 594 (red) conjugated goat anti-rabbit IgG. Merged images of differential interference contrast (DIC) and fluorescence microscopy are shown on the right. All slides were incubated with polyclonal FP-2 together with either plasmepsin IV (C) or LWL1 (D) monoclonal antibodies followed by Alexa-488-conjugated (green) goat anti-rabbit IgG and Alexa 594-conjugated (red) goat anti-mouse IgG. Merged images of green and red micrographs are shown on the right.

age site, a segment of human erythrocyte ankyrin predicted to contain the cleavage site was expressed as a His-tagged protein and incubated with FP-2 and the resulting digestion products were analyzed by MALDI-MS. This analysis revealed that the cleavage site lies immediately after arginine 1,210 of ankyrin. This proposed cleavage site is consistent with the previous observation that both native and recombinant FP-2 show a strong preference for a hydrophobic residue at the P2 position (15). Moreover, amino acids in the vicinity of this cleavage site are also consistent with the known substrate specificity of other papain family enzymes. Most importantly, arginine or lysine at the P1 position, a hydrophobic residue at the P2 position, and phenylalanine at the P1’ position are the preferred amino acids (20). Based on the mapping of the cleavage site, we designed a 10-mer peptide (AnkP) containing the cleavage site that completely inhibited the enzyme activity in vitro and abolished all of the known functions of FP-2, namely the cleavage of ankyrin, protein 4.1, and hemoglobin. It is noteworthy here that the AnkP peptide is at least 50-fold more potent in inhibiting FP-2 than the protein 4.1 peptide (*K* for AnkP is ~2 μM compared with ~100 μM for the protein 4.1 peptide) identified earlier (10). This finding suggests that ankyrin might be a preferred substrate of FP-2 in vivo.

To determine the effect of AnkP on the growth and development of *P. falciparum*, we utilized a recent technique where peptides were delivered into intact RBCs by fusion with the Antennapedia homeoprotein internalization domain (22). Human erythrocytes infected with stage-specific parasites were incubated with 100–250 μM of the fused peptide (Ant-AnkP). Giemsa staining of thin smears after 6 and 24 h of incubation showed that although the ring-stage parasites matured normally, the development of trophozoites and schizonts was markedly inhibited. <10% of new ring-stage parasites were detected compared with control cultures containing the same final concentrations of Antennapedia peptide, AnkP peptide, or Me$_3$SO. It is noteworthy here that although the Ant-AnkP peptide is likely to inhibit the hemoglobinase activity of FP-2, incubation with trophozoite-infected RBCs did not result in the formation of enlarged food vacuole, a feature indicative of the accumulation of undegraded hemoglobin generally observed with leupeptin (26, 27). This inconsistency could be attributed to the fact that leupeptin is a relatively membrane-impermeable cysteine protease inhibitor and enters the infected RBC via the parasite-induced permeation pathways (28) and therefore preferentially targets the intracellular parasite. In contrast, the Ant-AnkP peptide presumably enters by fusing with the erythrocyte membrane. Consistent with this view, our recent unpublished studies have shown that in the presence of a membrane-permeable cysteine protease inhibitor MDL 28170, an enlarged food vacuole is not formed yet the parasite release is completely blocked (data not shown). Together, our studies suggest that the route of entry of synthetic inhibitors influence their targeting efficiency to different compartments within the parasitized RBCs, thus giving rise to distinct modes of inhibitory functions.

Our results, as presented in this paper, identify the falcipain-2 cleavage site within ankyrin and demonstrate that a specific peptide derived from the COOH-terminal end of the spectrin-binding domain of ankyrin could effectively block the late-stage malaria parasite development in RBCs. Previous studies have shown that the erythrocyte ankyrin is also a substrate of γ-calpain, an intracellular cysteine protease (29). Although γ-calpain has been recently shown to be dispensable for malaria parasite development (30), we reasoned that if AnkP inhibited the γ-calpain activity then the observed inhibitory effect of the peptide on intracellular parasite development may not reflect the sole contribution of FP-2. To test this possibility, we compared the cleavage patterns of ankyrin obtained after digestion with either FP-2 or γ-calpain. Spectrin-actin-depleted erythrocyte IOVs were incubated with either rFP-2 (10) or with γ-calpain (31) followed by SDS-PAGE and immunoblot analysis. We found that the FP-2 and γ-calpain cleave ankyrin at different sites, producing distinct cleavage products (data not shown). Thus, it is unlikely that the ankyrin
peptide inhibits parasite growth by inhibiting the host \( \mu \)-calpain activity and is consistent with the normal parasite life cycle in \( \mu \)-calpain null mice (30).

Finally, to address the issue of whether the erythrocyte membrane skeleton serves as a physiologically relevant target of FP-2, we investigated the cellular localization of the parasite enzyme within infected erythrocytes. Confocal fluorescence microscopy using affinity-purified FP-2 antibodies provided clear evidence of the possible proximity between the FP-2 enzyme and the membrane skeleton during late stages of parasite maturation. Affinity-purified FP-2 antibodies, which specifically recognize both the mature and the proenzyme, showed that the FP-2 is present in the food vacuole, cytosol, and in the vesicle-like structures within the host cytosol. These vesicle-like structures were seen originating from the PVM and reaching to the periphery of the host plasma membrane. Because the native FP-2 in the trophozoite extract is predominantly the \( \sim 27\)-kDa mature form (Fig. 5, lane 3), it is likely that these vesicles contain the mature active enzyme. Furthermore, the co-labeling experiments with the LWL1 antibody suggest that while the LWL1 staining completely overlaps with the FP-2 staining, there are additional areas of FP-2 staining that are distinct from the tubovesicular network yet localized to the vesicles in the erythrocyte cytoplasm. This unique staining pattern of FP-2 suggests that it may be transported to the host membrane via these vesicles. Furthermore, as predicted for type II membrane proteins, it is possible that the active domain of FP-2 is extruded from these vesicles, thus permitting proteolysis of erythrocyte skeletal proteins without the necessity of the enzyme being released from the vesicles.

Considering the cleavage site specificity of FP-2 and the intense staining as well as the location of the parasite enzyme, our results strongly suggest that the FP-2 function is not necessarily restricted to hemoglobin degradation during its intracellular life cycle. The presence of large amount of FP-2 outside of the food vacuole and near the erythrocyte membrane skeleton is consistent with the proposed involvement of ankyrin and protein 4.1 cleavage in the merozoite release. Future exploration of the trafficking mechanism of FP-2 may lead to the development of specific inhibitors of parasite release from red blood cells.

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