

Food vacuole plasmepsins are processed at a conserved site by an acidic convertase activity in *Plasmodium falciparum*

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Abstract

Intraerythrocytic *Plasmodium falciparum* digests vast amounts of hemoglobin within an acidic food vacuole (FV). Four homologous aspartic proteases participate in hemoglobin degradation within the FV. Plasmepsin (PM) I and II are thought to initiate degradation of the native hemoglobin molecule. PM IV and histo-aspartic protease (HAP) act on denatured globin further downstream in the pathway. PM I and II have been shown to be synthesized as zymogens and activated by proteolytic removal of a propeptide. In this study, we have determined that the proteolytic processing of FV plasmepsins occurs immediately after a conserved Leu-Gly dipeptidyl motif with uniform kinetics and pH and inhibitor sensitivities. We have developed a cell-free in vitro processing assay that generates correctly processed plasmepsins. Our data suggest that proplasmepsin processing is not autocatalytic, but rather is mediated by a separate processing enzyme. This convertase requires acidic conditions and is blocked only by the calpain inhibitors, suggesting that it may be an atypical calpain-like protease.

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1. Introduction

Malaria is one of the world's deadliest infectious diseases, killing nearly 2 million people, mostly children, annually [1]. *Plasmodium falciparum*, the parasite that causes the most lethal form of malaria, resides for much of its life cycle within the host erythrocyte. Intraerythrocytic stages of *P. falciparum* rapidly consume almost all of the red blood cell hemoglobin and utilize it as a nutrient source during growth and maturation [2]. Hemoglobin catabolism is essential for parasite survival; agents that disrupt this process cause parasite death both in culture and in animal models [3,4]. It is of great interest to understand the molecular details of hemoglobin catabolism, as inhibitors of this pathway are good prospects for antimalarial chemotherapy.

Intracellular parasites ingest hemoglobin through the cytotome, a pear-shaped structure formed by invagination of the parasitophorous vacuolar membrane and parasite plasma

membrane [5–8]. In *P. falciparum*, hemoglobin-laden transport vesicles surrounded by two membranes are believed to bud from the cytotome and fuse with an acidic, degradative organelle called the food vacuole (FV) [7,8].

In the *P. falciparum* FV, several proteases act in a semi-ordered pathway to degrade hemoglobin [2,9]. Two aspartic proteases, plasmepsin (PM) I and II, appear to initiate the degradative process by cleaving a conserved hinge region in the α chain of native hemoglobin [10]. Several proteases are then capable of acting on denatured or fragmented globin. These include two cysteine proteases called falcipain-2 and -3 [11–13] and two plasmepsin paralogs, histo-aspartic protease (HAP) and PM IV [14–16]. HAP and PM IV are approximately 60% identical to PM I and II at the amino acid level [17]. HAP, briefly known as PM III, is an active enzyme despite having an unusual active site with a histidine in place of one of the two standard catalytic aspartates [16]. Further downstream in the pathway a metalloprotease, falcilysin, acts on 15–20 amino acid globin fragments to generate small peptides [18]. These peptides may be exported out of the FV for terminal degradation to amino acids in the parasite cytosol [19].

The biosynthesis of PM I and II has been previously studied [20]. Each proenzyme is synthesized as a 51 kD type II integral membrane protein containing a transmembrane

Abbreviations: PM, plasmepsin; HAP, histo-aspartic protease; FV, food vacuole; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ALLM, *N*-acetyl-Leu-Leu-methioninal

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domain within its prosegment. Following proteolytic removal of the prosegment, a 37 kD active, soluble enzyme is formed. In vivo pulse-chase studies using specific PM I and II antibodies revealed that both proenzymes are processed rapidly, with a $t_{1/2}$ of 20 min. In culture, processing is blocked by pH disrupting agents (bafilomycin and chloroquine) and tripeptide aldehydes, *N*-acetyl-Leu-Leu-norleucinal (ALLN) and *N*-acetyl-Leu-Leu-methioninal (ALLM), which inhibit proteasomes and a variety of cysteine proteases [20]. Whether these compounds act directly or indirectly to inhibit processing could not be addressed in the cultured parasite system.

In this study, we have characterized the biosynthesis of the newly identified plasmepsin paralogs, HAP and PM IV, defined the site at which three of the four proplasmepsins are processed in vivo, and have developed a cell-free assay that generates correctly processed mature plasmepsins. Our data implicate a calpain-like convertase in the processing of all four proplasmepsins. The convertase is an attractive drug target because its inhibition has potential to disrupt the entire hemoglobin degradation pathway.

2. Materials and methods

2.1. Reagents

All reagents were obtained from Sigma, St. Louis, MO, except for calpain inhibitors IV (CI IV; Z-Leu-Leu-Tyr-FMK) and V (CI V; morpholinoureidyl-Val-homophenylalanyl-FMK (Mu-Val-HPH-FMK)) (Calbiochem), Brefeldin A (BFA, Calbiochem), pepstatin A (ICN), and [3 H]leucine, [3 H]isoleucine, and [35 S]Protein Express (NEN-Dupont).

2.2. Antibodies

All anti-plasmepsin antibodies were produced and determined to be monospecific as described [16,20]. Antibodies 574 and 737 are polyclonal; antibodies 3E10-19 and 13.9.2 are monoclonal.

2.3. Culture of parasites

Plasmodium falciparum clone HB3 (a gift of Dr. W. Trager) was grown at 37 °C under 3% O₂, 3% CO₂, 94% N₂ in RPMI 1640 medium supplemented with 25 mM HEPES, 30 mg/l hypoxanthine, 0.225% NaHCO₃, and 0.5% Albumax I (Life Technologies). Parasites were grown in 2% human red blood cells and were synchronized using sorbitol treatment [21].

2.4. In vitro translation

Capped plasmepsin RNA was prepared using linearized plasmid DNA and T7 polymerase Message Machine kit (Ambion) following the manufacturer's instructions. Transcript was added to nuclease-free rabbit reticulocyte lysate

(Promega) in the presence of [35 S]methionine-cysteine and 10 µg brewer's yeast tRNA per 25 µl translation reaction.

2.5. Immunoprecipitation

Immunoprecipitations were done using denaturing conditions. In vitro translated protein or isolated *Plasmodium* parasites were resuspended in 1% SDS, boiled for 5 min, and then centrifuged for 10 min at 13,000 × *g* to pellet debris. The supernatant was transferred to a fresh tube and mixed with IP buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% deoxycholate, 1% Triton X-100), antibody, and 70 µl of 50% slurry of Protein A (PM I, II, and HAP) or Protein G (PM IV) sepharose (Pharmacia) and incubated for 1 h at 4 °C. Beads were washed 3 × in IP buffer (without deoxycholate), 1 × in 1 M NaCl, and 1 × in 150 mM NaCl. Non-reducing sample buffer was added and samples were boiled, and analyzed by 10% SDS-PAGE and fluorography. Antibodies recognizing PM I (574), II (737), and HAP (3E10-19) were added at a dilution of 1:200. PM IV antibody 13.9.2 was added at a dilution of 1:4.

2.6. Pulse-chase experiments

Synchronized cultures of trophozoite-infected RBCs were washed once in RPMI without methionine and cysteine, resuspended in labeling medium (RPMI without methionine and cysteine, supplemented with glutamine (0.3 mg/ml), Albumax I (5 mg/ml), glucose (2 mg/ml), hypoxanthine (20 µg/ml), thymidine (2 µg/ml), sodium pyruvate (100 µg/ml), HEPES (6 mg/ml), sodium bicarbonate (0.5 mg/ml) containing 140 µCi/ml [35 S]methionine-cysteine (Protein Express, NEN-Dupont), and incubated at 37 °C for 5 min. Cultures were chased by replacing the labeling media with complete RPMI containing 10 µg/ml cycloheximide and incubating for various times at 37 °C. For inhibitor experiments, inhibitors were added during the pulse and the chase, except where noted. Isolated *Plasmodium* parasites were harvested with chilled buffers and stored at -70 °C until processed.

2.7. Radiosequencing

Trophozoite-stage parasites were washed briefly in labeling media (RPMI deficient in either leucine or isoleucine; Sigma), and then resuspended in labeling media with either [3 H]isoleucine (100 µCi/ml) or [3 H]leucine (200 µCi/ml) and incubated for 2–3 h at 37 °C. Denaturing immunoprecipitations were performed as described above with specific anti-plasmepsin antibodies. Immunoprecipitates were fractionated by SDS-PAGE and blotted onto PVDF membranes. Plasmepsin bands were excised and subjected to Edman degradation. Each cycle from the sequencer was assayed for radioactivity in a scintillation counter. Non-specific background increases with successive cycles as is typical with Edman degradation.

2.8. In vitro processing assays

Trophozoite parasites were radiolabeled as described above except that BFA (5 µg/ml) and [³⁵S]methionine-cysteine were added at the same time. Cultures were incubated for 4–5 h and then harvested and lysed in PBS by gentle sonication. Radiolabeled lysates were incubated in the presence of an equal amount of unlabeled lysate and various inhibitors in 100 mM sodium acetate buffer, pH 5.0, for 3–4 h at 37 °C. Reactions were stopped by adding an equal volume of 2% SDS and boiling. Specific plasmepsins were immunoprecipitated as described above. To determine the pH sensitivity of the convertase, lysates were incubated in 100 mM citrate–phosphate buffers from pH 4 to 6.5.

2.9. Immunofluorescence microscopy

Thin smears of infected RBCs were made on coverslips, allowed to air dry, and fixed in acetone for 10 min at RT. Samples were then washed, permeabilized in 0.2% Triton X-100 in PBS for 5 min at RT, and blocked for 30 min at RT in PBS/0.2% Tween-20/1% goat serum. Samples were immunolabeled with mouse anti-PM IV (13.9.2, undiluted hybridoma supernatant) or rabbit anti-BiP (1:1000) followed by Alexa-conjugated goat anti-mouse IgG or Oregon Green-conjugated goat anti-rabbit IgG. Coverslips were mounted in Vectashield containing DAPI (Vector Laboratories) and viewed immediately using a Zeiss axiovision microscope, camera, and software and exported to Photoshop for manipulation.

3. Results

3.1. Plasmepsin antibodies are specific

Anti-plasmepsin antibodies were used to immunoprecipitate in vitro translated plasmepsins under denaturing conditions (Fig. 1). Each antibody used is specific for a single plasmepsin.

3.2. PM I, II, IV, and HAP are secretory pathway proteins processed with similar kinetics

To compare the biosynthesis of PM I, II, IV, and HAP, intraerythrocytic *P. falciparum* trophozoites were pulse-labeled with [³⁵S]methionine-cysteine and chased in unlabeled complete medium. Processing was monitored by immunoprecipitation with specific plasmepsin antibodies. The kinetics of processing of all four plasmepsins is similar to our previously reported results for PM I and II [20] (Fig. 2). Processing is largely complete within 30 min.

The fungal metabolite BFA causes accumulation of newly synthesized secretory proteins in a fused ER/Golgi compartment. In parasites treated with BFA, processing of PM I and II [20] and HAP (data not shown) was prevented, suggesting

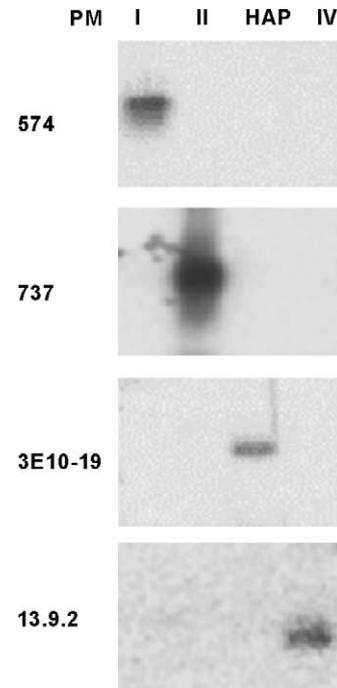


Fig. 1. Specificity of selected antibodies in immunoprecipitation assays. In vitro translated proforms of PM I, II, IV, and HAP (51 kD) were prepared and used as substrates for immunoprecipitation using antibodies specific for PM I (574), PM II (737), HAP (3E10-19), and PM IV (13.9.2).

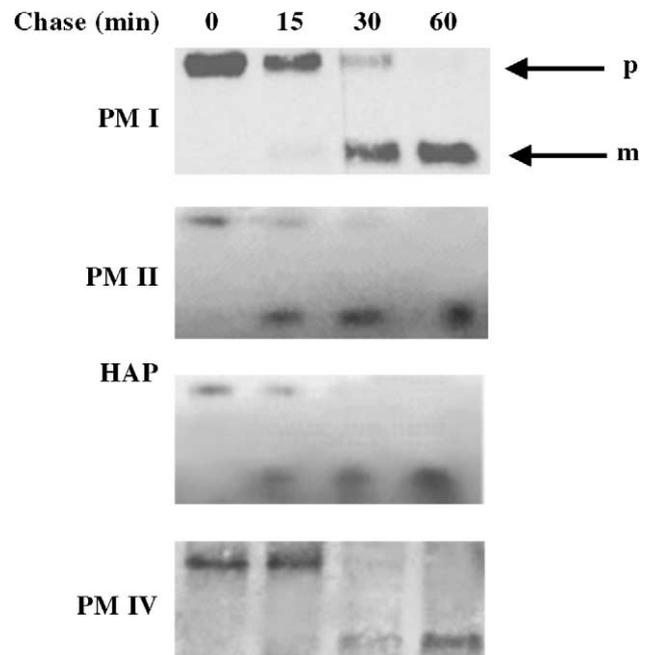


Fig. 2. PM I, II, IV, and HAP are processed with similar kinetics. Trophozoites were pulse-labeled with [³⁵S]methionine-cysteine for 5 min, chased in unlabeled medium for the time indicated, and immunoprecipitated with anti-plasmepsin antibodies. The 51 kD proform (p) and 37 kD mature form (m) are indicated.

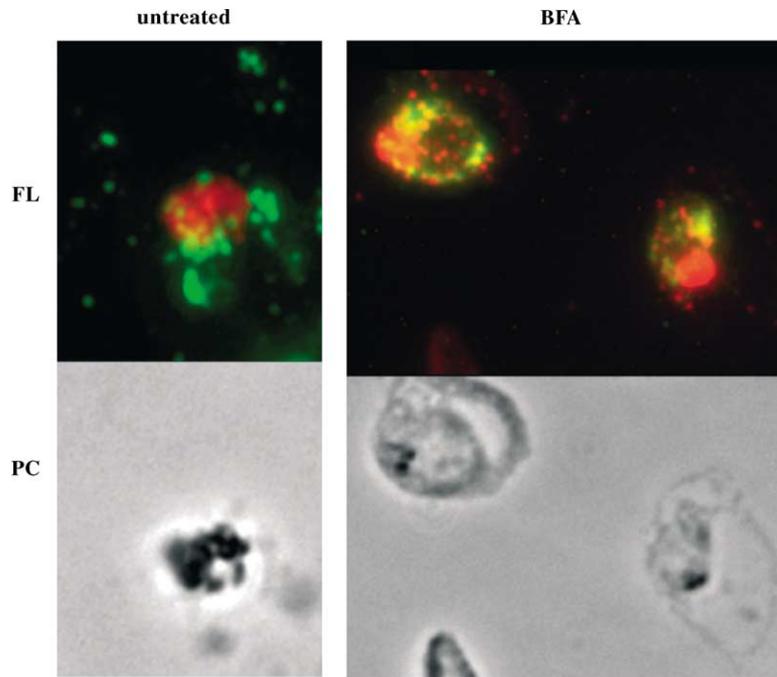


Fig. 3. Plasmepsin trafficking through the secretory pathway is sensitive to BFA. Trophozoites were stained for PM IV (red) and Bip (green). Colocalization of the proteins appears yellow. Representative fluorescence (FL) and phase contrast (PC) images are shown. Hemozoin is visible as dark crystals in the PC images.

that the plasmepsins traffic through the secretory pathway. Immunofluorescence microscopy indicates that BFA disrupts plasmepsin transport through the secretory pathway (Fig. 3). After a 45 min incubation with BFA, a portion of PM IV (presumably newly synthesized protein) is retained in the ER where it colocalizes with the ER marker BiP (Fig. 3). PM IV staining is also seen over the FV in BFA-treated cells (marked by pigmented hemozoin in phase image), presumably reflecting previously synthesized protein. In untreated cells there is no colocalization of PM IV and BiP.

3.3. *In vivo* processing site of food vacuole plasmepsins is conserved

The site at which the proplasmepsins are processed *in vivo* has been unclear. Mature PM I and II have been purified from parasite FVs and their apparent processing sites determined by N-terminal amino acid sequencing [10]. It was puzzling that recombinant proplasmepsin II produced in *Escherichia coli* autoprocesses at a site 12 amino acids upstream of the N-terminus of purified native enzyme [22]. It was possible that the N-termini of the isolated native protein could have resulted from spurious proteolysis during purification. Alternatively, autoproteolysis of recombinant proenzyme could be a fortuitous *in vitro* artifact.

To determine where the proplasmepsins are processed *in vivo*, radiosequencing was performed on mature PM I, II, and HAP. Intraerythrocytic trophozoites were incubated with [³H]leucine or [³H]isoleucine, the appropriate plasmepsin was immunoprecipitated and subjected to Ed-

man degradation, and the radioactivity released in each cycle was determined (Fig. 4A). PM I showed a clear peak of [³H]leucine at cycle 8, PM II showed peaks of [³H]isoleucine at cycles 6 and 14, and HAP showed peaks of [³H]leucine at cycles 8, 11, and 15. These data are consistent with the *in vivo* processing occurring within a conserved sequence in all three proteins: (Y/H)LG* (S/N)XXD (Fig. 4B). This sequence is also present at the identical region in PM IV. Sufficient amounts of PM IV could not be immunoprecipitated for radiosequencing studies. However, the protein sequence and biosynthetic properties are very similar among these paralogs, suggesting that PM IV is most likely cleaved at the conserved processing site. The previously reported N-termini of mature PM I and II were identical to those determined by radiosequencing, arguing against artifactual trimming during protein purification in the earlier studies.

3.4. Processing is inhibited only by calpain inhibitors

We have previously reported the effects of numerous protease inhibitors on plasmepsin processing in culture [20]. The only protease inhibitors that prevent proplasmepsin processing in culture are the tripeptide aldehydes ALLN and ALLM (IC₅₀ = 5 μM). These compounds, known as calpain inhibitors, block the calpain family of cysteine proteases as well as other cysteine proteases and proteasomes. Importantly, proplasmepsin processing was not blocked by EGTA, or general inhibitors of cysteine proteases (E64, leupeptin) and proteasomes (lactacystin) [20].

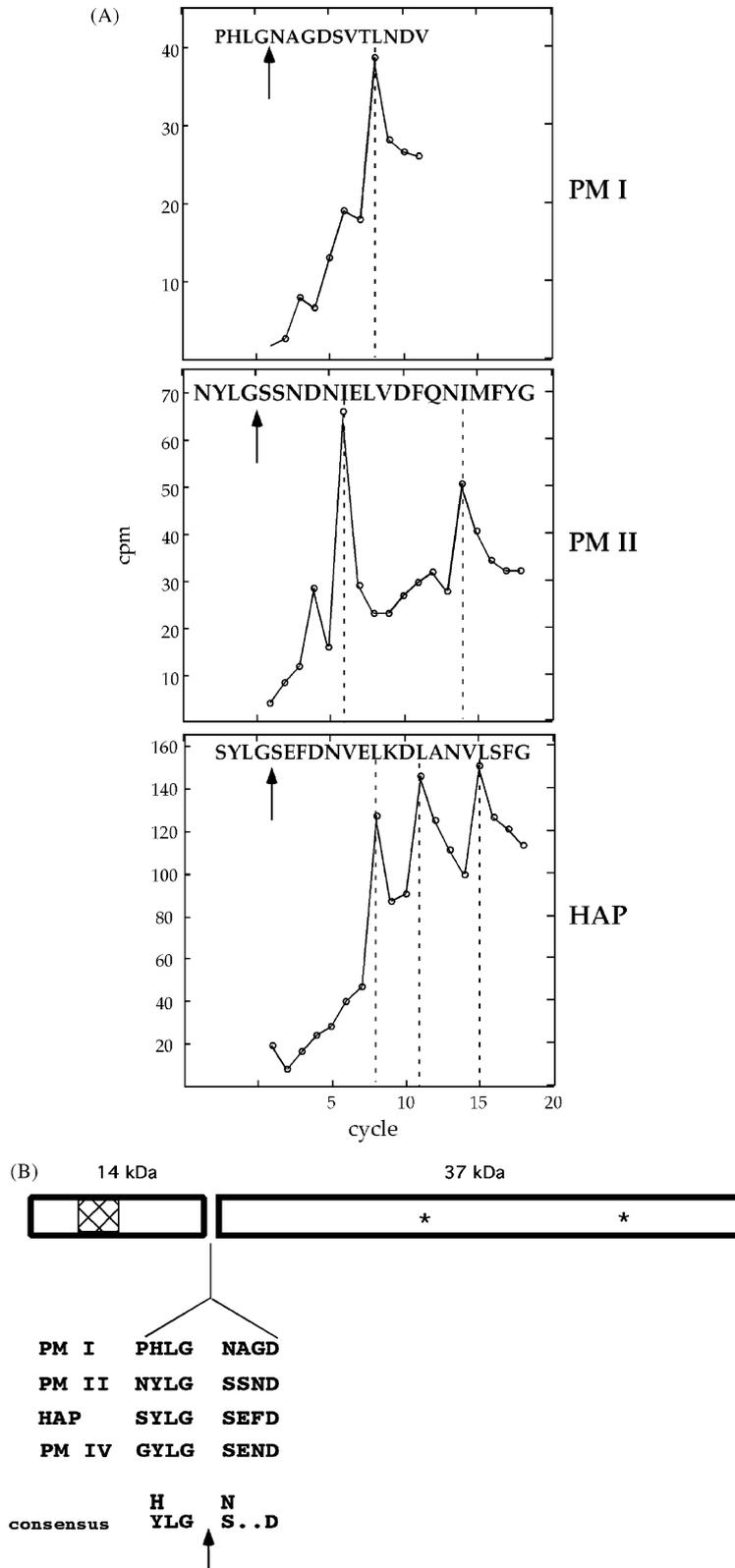


Fig. 4. Proplasmepsins are cleaved at a conserved site in vivo. (A) Mature PM I, II, and HAP were immunoprecipitated from metabolically labeled trophozoites and subjected to multiple cycles of Edman degradation. The radioactivity recovered at each cycle is plotted. Amino acid sequences shown at the top correspond to plasmepsin sequences surrounding the cleavage site (arrow). PM I and HAP were labeled with [³H]leucine; PM II was labeled with [³H]isoleucine. (B) Schematic of conserved structure and cleavage site motif in plasmepsins. Each piece is of 14 kDa and contains a transmembrane domain (hatched box). Each mature enzyme is of 37 kDa and contains two active site motifs (*). The conserved sequence surrounding the cleavage site (arrow) of each plasmepsin is indicated. The cleavage site in PM IV is proposed, but not confirmed.

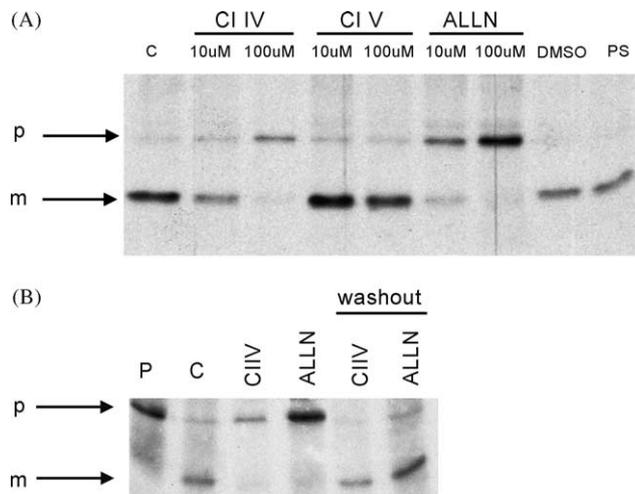


Fig. 5. Inhibitor sensitivity of proplasmepsin processing in culture. (A) Trophozoites were pulse-labeled with [³⁵S]methionine-cysteine for 5 min and chased for 1 h in the presence of calpain inhibitor IV (CI IV), calpain inhibitor V (CI V), ALLN, pepstatin (PS, 100 μM), or DMSO. Plasmepsins were immunoprecipitated. The 51 kD pro (p) and 37 kD mature (m) forms are shown. C, chase. Data shown are for PM I. Results were similar for PM II and HAP. (B) Inhibition of proplasmepsin processing by calpain inhibitors is reversible. Trophozoites were pretreated with inhibitors, pulse-labeled with [³⁵S]methionine-cysteine for 5 min and chased for 1 h in complete media containing cycloheximide (10 μg/ml) and protease inhibitors or for 3 h in media containing cycloheximide, but lacking protease inhibitors (washout). Data for immunoprecipitated PM I are shown. Results were similar for PM II and HAP. Calpain inhibitors assayed are ALLN (100 μM) and CI IV (100 μM). P, pulse; C, chase; p, proform; m, mature form.

To determine if other calpain inhibitors affect processing, trophozoite-stage parasites were pulse-labeled with [³⁵S]methionine-cysteine and chased in the presence of several calpain inhibitors plus cycloheximide. Processing was inhibited by ALLN and CI IV (Z-Leu-Leu-Tyr-FMK), but not by CI V (Mu-Val-HPh-FMK) (Fig. 5A). As with ALLN, the inhibition by CI IV was reversible when the cultures were chased for 3 h in the absence of inhibitor (Fig. 5B). This was surprising given that CI IV is a fluoromethyl ketone which usually binds irreversibly [23]. Perhaps this inhibitor binds to the active site in an orientation that is not optimal for interaction with the reactive group. Reversibility was not due to new enzyme synthesis since the experiment was done in the presence of cycloheximide.

3.5. *In vitro* processing assay

In the parasite culture system, some inhibitors may not block proplasmepsin processing because they are membrane impermeable and cannot accumulate to high levels intracellularly. Others may block processing indirectly through effects on protein transport or other cellular functions. To avoid permeability and transport issues and to probe the true inhibitor sensitivity of the convertase, it was necessary to develop a cell-free processing assay. Assays utilizing peptides, recombinant plasmepsins, or *in vitro* translated plasmepsins

as substrates all generated products cleaved at incorrect sites by proteases with incorrect inhibitor sensitivities (not shown). However, an assay using native membrane-bound proenzyme as substrate yielded correctly processed mature plasmepsin. In this assay, trophozoites were radiolabeled with [³⁵S]methionine-cysteine in the presence of BFA. This yielded a pool of radiolabeled proplasmepsins. The labeled cells were lysed by gentle sonication and the lysates were incubated at 37 °C. The processing of specific plasmepsins was assessed by immunoprecipitation.

Processing occurred between pH 4 and 5.5 in the cell-free assay (Fig. 6A). This is consistent with the finding that bafilomycin and chloroquine, agents that increase vacuolar pH, inhibited processing in culture [20]. Cleavage occurred at the *in vivo* processing site in this cell-free assay, as determined by radiosequencing (Fig. 6B). ALLN, ALLM, and CI IV inhibited processing in the cell-free assay while other protease inhibitors did not (Fig. 6C). The concordance of pH optimum, inhibitor sensitivity, and cleavage site *in vitro* and *in culture* validate the use of this cell-free assay.

4. Discussion

PM I, II, and HAP are processed under acidic conditions, with similar kinetics, at a conserved site. We suggest that PM IV is also cleaved at the conserved processing site because of its similar protein sequence and biosynthetic properties. The inhibitor sensitivity of processing suggests that proplasmepsin activation is not autocatalytic, but rather is mediated by a novel convertase. The aspartic protease inhibitor, pepstatin A, inhibits activity of all four plasmepsins, but does not block proplasmepsin processing. Conversely, ALLN is a poor inhibitor of plasmepsin activity, but completely blocks proplasmepsin processing.

While most aspartic proteases undergo autocatalytic activation, there is an increasing number of examples of processing *in trans* by other enzymes [24]. The existence of a prorenin activating enzyme has been known for many years, although its identity remains controversial [25–29]. Activation of BACE, the β-secretase involved in Alzheimer's disease pathology, is thought to be mediated by a furin-like protease in the Golgi [30].

It is unclear why acidic conditions are required for proplasmepsin processing. In the proplasmepsins, the cleavage site at the pro-mature junction is within a surface-exposed, tight loop known as the Tyr-Asp loop [31]. As most proteases prefer to cleave unstructured substrates, acidification may be needed to disrupt salt bridges in the loop and generate an extended conformation that can be cleaved by the convertase. Alternatively, it might be that the processing site is always exposed, but that convertase catalysis is optimal at acidic pH [31].

It is not clear where proplasmepsin activation occurs. The plasmepsins remain in their proforms in the presence of BFA suggesting that processing occurs downstream of the ER or

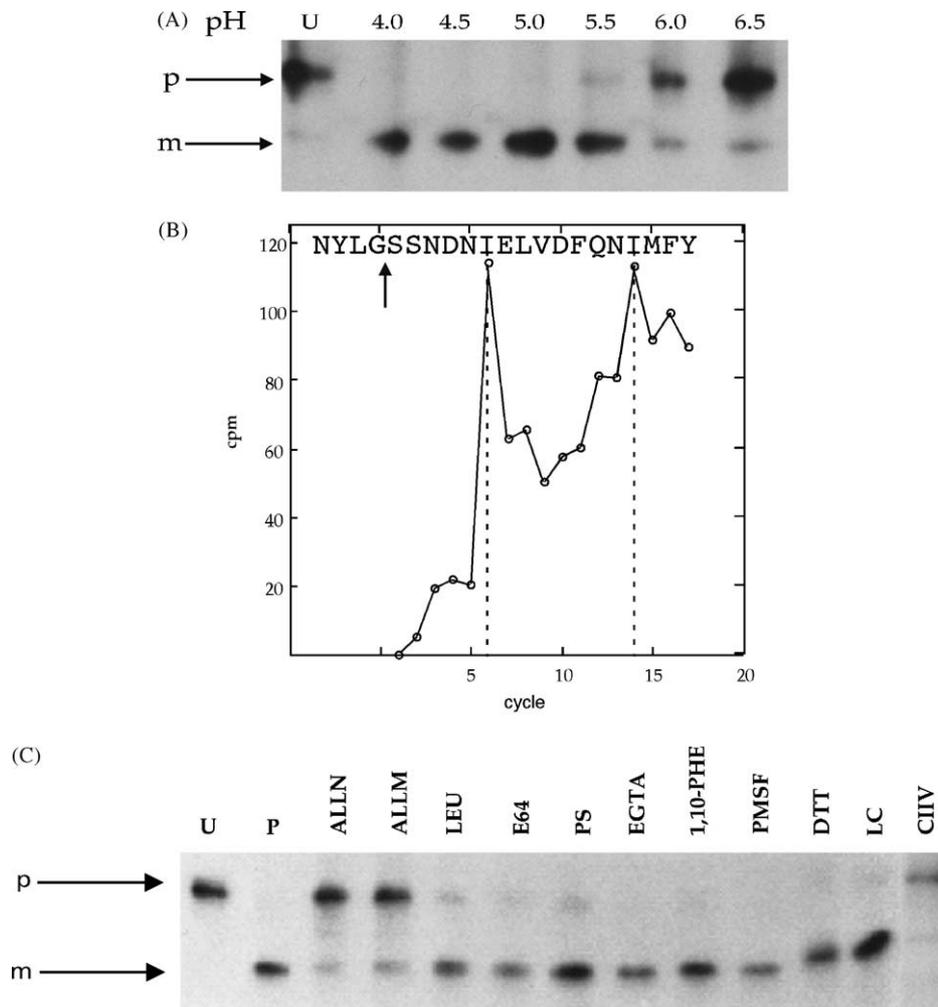


Fig. 6. Convertase properties in an in vitro processing assay parallel those in culture. (A) pH sensitivity of proplasmepsin processing in the in vitro assay. Trophozoites were incubated with [35 S]methionine-cysteine and BFA (5 μ g/ml) for 7.5 h. Parasites were harvested and lysed by three cycles of freeze–thawing. Radiolabeled lysates were incubated with additional non-labeled lysate and citrate–phosphate buffer at indicated pH for 4.5 h. Samples were immunoprecipitated with anti-PM II antibody 737. Identical results were obtained for PM I and HAP (not shown). p, proform; m, mature form; U, unprocessed. (B) PM II cleavage site in the in vitro processing assay. Trophozoites were radiolabeled with [3 H]isoleucine. In vitro cleavage assays were set up as described in Fig. 5A at pH 5.0, and PM II was immunoprecipitated. Immunoprecipitated protein was subjected to multiple rounds of Edman degradation. The radioactivity of each cycle is plotted. Processing occurs at the arrow. The N-terminus of in vitro generated mature PM II matched that of in vivo processed PM II (shown at the top). (C) Inhibitor sensitivity of proplasmepsin processing in the in vitro assay. Trophozoites were metabolically labeled in the presence of BFA and lysed. Radiolabeled lysates were incubated in 100 mM sodium acetate, pH 5.0, with additional non-labeled lysate and inhibitors. Samples were immunoprecipitated with PM I-specific antibody 574. Results were similar for PM II and HAP. Inhibitors assayed are: ALLN, ALLM (15 μ M), leupeptin (10 μ M), E64 (10 μ M), pepstatin (10 μ M), EGTA (5 mM), 1,10-phenanthroline (1 mM), PMSF (1 mM), DTT (10 mM), lactacystin LC, 10 μ M, CI IV (50 μ M). U, unprocessed; P, processed; p, proform; m, mature form.

Golgi. The acidic pH optimum of processing suggests that activation occurs in the FV or in acidic transport vesicles en route to the FV. This is analogous to many mammalian proteases whose prodomains are cleaved in late endosomes or lysosomes [32].

The proplasmepsin convertase is sensitive to several calpain inhibitors (ALLN, ALLM, CI IV), but is unaffected by general cysteine protease inhibitors or calcium concentration. This unusual inhibitor profile suggests that the convertase may be an atypical calpain. Several atypical calpains are blocked by calpain inhibitors [33–35], but are insensitive to E64 [36,37] as well as to calcium [38,39]. This is

in contrast to typical calpains that are neutral cysteine proteases activated by calcium and inhibited by E64 and EDTA [40]. Furthermore, like the proplasmepsin convertase, calpains tend to process rather than degrade proteins [40] and their substrates are commonly membrane proteins [36,41]. The *P. falciparum* genome encodes a single calpain gene (www.plasmodb.org), suggesting that the calpain has an important, non-redundant function and is a good target for drug development. Additional characterization of the *P. falciparum* calpain is required to establish a role in plasmepsin maturation. An alternative hypothesis consistent with our data is that the calpain may act upstream of the convertase.

We have developed a specific cell-free processing assay that will be useful in characterization of the convertase. The convertase has great potential as an antimalarial drug target. Because of its position at the beginning of the hemoglobin degradation pathway, blocking the convertase should result in simultaneous inhibition of PM I, II, IV, and HAP. Such a drastic blockade of hemoglobin degradation is likely to be lethal to the malaria parasite.

Acknowledgements

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