

Short communication

## *Plasmodium falciparum* falcilysin: an unprocessed food vacuole enzyme

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Five hundred million infections and nearly two million deaths each year are attributed to the protozoan *Plasmodium falciparum*, a causative agent of human malaria. With the increasing prevalence of drug resistant strains, there is an urgent need to identify new drug targets. Examination of the parasite's unique metabolic pathways, such as hemoglobin degradation, provides candidates for chemotherapeutic development.

Intraerythrocytic development of the parasite is dependent upon degradation of red blood cell hemoglobin. A semi-ordered pathway of proteases mediates this catabolism, which occurs in the acidic organelle called the food vacuole. The aspartic proteases, plasmepsins I and II, are proposed to be responsible for initial cleavage of hemoglobin in a conserved hinge region of the alpha chain [1,2]. Plasmepsins, and a family of cysteine proteases, falcipain-2 and 3, then carry out further degradation of the denatured globin [2–5]. The resulting small globin peptides serve as substrates for falcilysin (FLN) [6].

FLN was first identified in 1999 when analysis of degraded globin fragments from the food vacuole revealed several peptides with cleavage sites that could not be attributed to the known proteases [7]. Specifically, a protease preferring to cleave substrates at polar or charged residues was implicated; this stands in contrast to the other hemoglobin-degrading proteases, which favor cleavage at hydrophobic residues. Purification of the native enzyme from food vacuoles revealed that it is a monomeric enzyme with a molecular weight of 130,000, by far the largest hemoglobin catabolic protease. FLN is a member of the M16 family of metalloproteases, enzymes character-

ized by an inverted active site motif, HXXEH, where the histidines coordinate a catalytic zinc ion [8]. Other M16 family members are also oligoendopeptidases whose substrates include physiologically important molecules such as insulin [9],  $\alpha$  factor [10,11], somatostatin [12], and transforming growth factor  $\alpha$  [13]. Chelators such as EDTA and 1,10-phenanthroline readily inhibit FLN [6].

Beyond these data, very little is known about FLN. Considering the role of this enzyme in a unique and essential metabolic pathway, it warrants further investigation. FLN may be a worthy drug target as it has been shown that inhibition of hemoglobin catabolism is lethal to parasites [14,15]. In this study, we show that FLN, unlike other food vacuole proteases, does not have a propeptide. It is targeted and functional without processing. We conclude that targeting information must reside within the mature enzyme.

To facilitate studies of FLN, we generated a rabbit polyclonal anti-peptide antiserum directed against a unique region of the protein's C-terminus. The affinity-purified antibody (A1219T) is monospecific and pre-bleed serum does not show reactivity to FLN (data not shown). FLN protein expression was determined via immunoblotting with lysates from parasites harvested over a time course of intraerythrocytic development (Fig. 1A). The 130 kDa band of FLN is first detected at 24 h post-invasion. This corresponds to the late ring, early trophozoite stage. FLN expression increases in the mid-trophozoite stage (31 h) and persists through the schizont stage. This expression profile is similar to that observed for other late-stage hemoglobin-catabolic proteases, such as HAP [3]. It also suggests that the enzyme may serve another purpose in mature schizonts, as the majority of hemoglobin degradation is already complete at schizogony. Both plasmepsin II and falcipain-2 have been shown to cleave host membrane proteins such as band 4.1 and spectrin [16,17]. The significance of these proteolytic events

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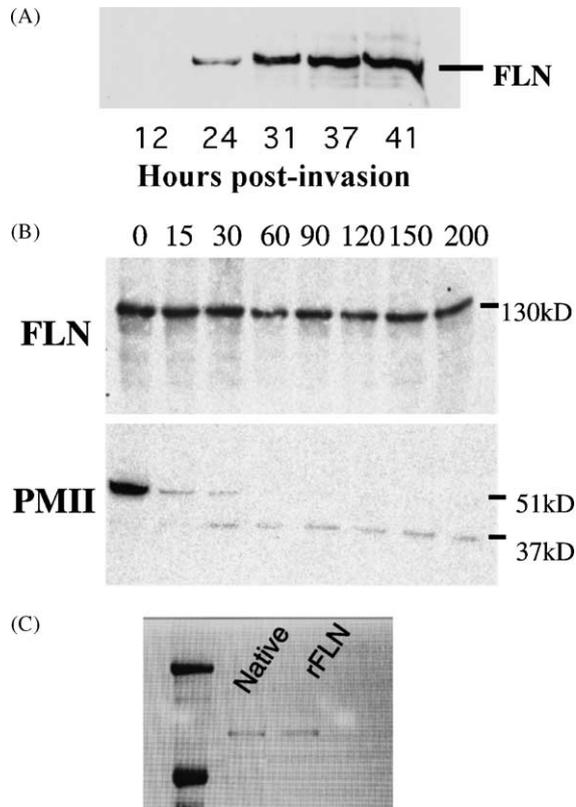


Fig. 1. FLN is not processed during targeting or maturation. (A) FLN is expressed from early trophozoite to schizont stages. Trophozoite lysate, which was derived from the *P. falciparum* clone HB3 (Honduras, gift of W. Trager, Rockefeller University) cultured using the methods of Trager and Jensen [27] in serum-free RPMI media supplemented with 0.5% Albumax (Gibco BRL, Grand Island, NY), was generated from parasites (3% parasitemia and 2% hematocrit) harvested by saponin lysis at multiple time points during the intraerythrocytic life cycle. A volume of total parasite lysate equivalent to 1.2 ml of culture was loaded into each lane, separated by SDS-PAGE, and immunoblotted with A1219T. The A1219T antibody was derived from polyclonal rabbit antiserum made with a synthetic peptide containing residues spanning a unique region of the FLN C-terminus (residues 899–984) linked to glutathione S-transferase (GST) and coupled to keyhole limpet hemocyanin using the Freund's adjuvant (CoCalico Biologicals, Reamstown, PA). Affinity purification was performed as previously described [28] and antibody A1219T used at a 1:1000 dilution. (B) Pulse labeling experiments utilized synchronized cultures of trophozoite stage parasites (between 24 and 32 h post-invasion). 70 ml of culture (5% parasitemia, 2% hematocrit) was first washed in methionine and cysteine-depleted RPMI medium before being resuspended in the same medium at a density of  $2.0 \times 10^8$  parasites/ml with [ $^{35}$ S]-methionine-cysteine (330  $\mu$ Ci/ml) (Perkin-Elmer, Boston, MA). After a 10-min pulse labeling at 37 °C, the culture was centrifuged at  $643 \times g$  for 5 min at 4 °C. The chase began when the pellets were resuspended in prewarmed complete RPMI. Culture was incubated in chase medium at 37 °C for 0–200 min, harvested, and the lysate used for immunoprecipitations [18] with Ab A1219T (FLN) or Ab 737 (PM II). SDS-PAGE and fluorography followed. The experiment was repeated three times and a representative result is presented. (C) The native enzyme was purified as previously described [6]. For expression of recombinant enzyme, the entire *fn* open reading frame (ORF) was cloned into the NcoI site of the pET-3d expression vector (Novagen, Madison, WI) by PCR from genomic DNA. FLN-pET-3d was transformed into BL21 (DE3) pLysS-Codon Plus RIL cells (Stratagene, La Jolla, CA). For expression, LB medium with 34  $\mu$ g/ml of chloramphenicol and 60  $\mu$ g/ml ampicillin was inoculated with a single colony. Induction was begun at OD<sub>600</sub> of 0.8

remains unclear, but it raises the possibility that FLN may also be acting upon other substrates in mature schizonts.

Other food vacuole proteases, specifically plasmepsin II and falcipain-2 [4,18], are synthesized as proforms. Their pro-peptides, which contain putative transmembrane domains believed to be involved in targeting to the food vacuole, are cleaved to release the mature, soluble enzyme [18]. To determine if FLN is similarly processed, we performed a pulse-chase experiment using radiolabeled trophozoites. As shown in Fig. 1B, the FLN signal remains a single band at 130 kDa. We were not able to immunoprecipitate any new, smaller bands, nor do we observe a diminution of the 130 kDa band. This suggests that FLN does not undergo processing as it targets to the food vacuole, a feature that makes it unique among known food vacuole proteases. In contrast, plasmepsin II (PMII) is initially synthesized as a 51 kDa proform that is cleaved to generate the 37 kDa mature form. Not only is FLN unprocessed by pulse-chase analysis, it also remains undegraded. There is no detectable loss of signal over the 200 min chase period. FLN is a very stable enzyme.

Further support for the notion that FLN is not processed was found when native FLN, purified from food vacuoles, and recombinant enzyme were analyzed side-by-side via SDS-PAGE. Both proteins exhibit the same electrophoretic mobility (Fig. 1C). Additionally, N-terminal sequence from the recombinant enzyme begins with SKTPEWI, 55 amino acids into the open reading frame. The Ser is directly preceded by a Met. Met<sup>54</sup> may be the initiating methionine; it could be removed after synthesis. The native enzyme has a blocked amino-terminus but work by Eggleston et al. [6] using endoproteinase Lys-C identified several internal peptide fragments. The earliest fragment, with the sequence TPEWI-HEK, directly overlaps the N-terminal sequence obtained

with the addition of 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). After 3 h, the bacteria were harvested and washed in cold 20 mM Tris-HCl pH 8.0 and resuspended in 20 mM bis-Tris pH 7.5 supplemented with 1% Triton X-100, 1 mM PMSF, 1  $\mu$ M pepstatin A, and 10  $\mu$ M E-64. 100  $\mu$ g/ml of fresh lysozyme and 1  $\mu$ l/4 ml DNaseI were then added and the resuspension was incubated at 30 °C for 15 min followed by sonication. The sonicate was centrifuged at  $17,500 \times g$  for 1 h at 4 °C. The supernatant was applied to a 12 ml column of DEAE Sepharose (Amersham Pharmacia, Piscataway, NJ) equilibrated with 20 mM bis-Tris pH 7.5. Bound proteins were eluted stepwise in 0.1–0.5 M NaCl in 20 mM bis-Tris pH 7.5. DEAE fractions containing rFLN were identified by testing for activity against the  $\alpha$ 74–75 fluorogenic peptide [6]. Active fractions were pooled and applied to a MonoS 5/5 column (Amersham Pharmacia, Piscataway, NJ) equilibrated in 20 mM MES pH 5.5. Bound proteins were eluted using a linear gradient from 0 to 0.8 M NaCl over 40 min. Active fractions were loaded onto a Mono Q 5/5 column (Amersham Pharmacia, Piscataway, NJ) equilibrated in 20 mM bis-Tris pH 6.5. Bound proteins were eluted using the same salt gradient established for MonoS. The active fractions were supplemented with 0.01% Tween 20, aliquotted and stored at –80 °C. Purified enzyme concentration was quantified with the Pierce BCA kit (Rockford, IL). Equivalent amounts of purified native and recombinant protein were loaded in each lane. Bands were visualized with silver staining [29].

with the recombinant enzyme. The Met<sup>54</sup>-Ser<sup>55</sup> fragment was most likely not detected as the endoproteinase cleaved at Lys<sup>56</sup> and such a small peptide would be lost in the sequencing analysis. Additionally, the *fln* ORF contains six lysines within the first 54 residues and the endoproteinase digestion did not detect any fragments before Thr<sup>57</sup>. Therefore, the endoproteinase Lys-C data reconciles well with the recombinant N-terminal sequencing data, given the size equivalence of native and recombinant enzyme.

This information, in conjunction with the pulse-chase data, supports the hypothesis that FLN is not processed within the parasite. It also raises several interesting questions about FLN's synthesis and targeting. The other known hemoglobin catabolic proteases have a cleavable pro-piece along with a signal peptide to direct the nascent protein into the classical secretory system. If Met<sup>54</sup> is truly the initiating methionine, this would suggest that FLN does not possess a signal peptide. Indeed, analysis of the enzyme's sequence by signal peptide prediction programs (SignalP V1.1 [19], SIGFIND [20]) fails to identify a potential signal sequence. FLN may possibly reach the food vacuole via an alternative targeting pathway, which does not require secreted proteins to enter the ER lumen [21–25]. It is also curious to note that both the parasite and *E. coli* seem to utilize Met<sup>54</sup>. Yet, *Plasmodium* proteins often use internal Met start sites [21], and the plasmepsins also initiate from an internal methionine in the pET system [26].

FLN is unique among food vacuole denizens for several reasons—its preference for cleavage of charged/polar amino acids instead of hydrophobic residues [6], its large size as compared to the mature forms of plasmepsins and falcipains (both under 50 kDa), and, as we report, the lack of a large pro-piece present in other food vacuole proteases. It should be noted that a *Plasmodium* sequence that is both necessary and sufficient for directing a protein to the food vacuole has not been identified. Further study of FLN is needed to delineate its food vacuole targeting sequence, which we suggest is contained within the mature enzyme.

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