A *Plasmodium falciparum* Dipeptidyl Aminopeptidase I Participates in Vacuolar Hemoglobin Degradation*

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Intraerythrocytic growth of the human malaria parasite *Plasmodium falciparum* requires the catabolism of large amounts of host cell hemoglobin. Endoproteolytic digestion of hemoglobin to short oligopeptides occurs in an acidic organelle called the food vacuole. How amino acids are generated from these peptides is not well understood. To gain insight into this process, we have studied a plasmodial ortholog of the lysosomal exopeptidase cathepsin C. The plasmodial enzyme dipeptidyl aminopeptidase 1 (DPAP1) was enriched from parasite extract by two different approaches and was shown to possess hydrolytic activity against fluorogenic dipeptidyl substrates. To localize DPAP1 we created a transgenic parasite line expressing a chromosomally encoded DPAP1-green fluorescent protein fusion. Green fluorescent protein fluorescence was observed in the food vacuole of live transgenic parasites, and anti-DPAP1 antibody labeled the food vacuole in parasite cryosections. Together these data implicate DPAP1 in the generation of dipeptidyl substrates from hemoglobin-derived oligopeptides. To assess the significance of DPAP1, we attempted to ablate DPAP1 activity from blood stage parasites by truncating the chromosomal DPAP1-coding sequence. The inability to disrupt the coding sequence indicates that DPAP1 is important for asexual proliferation. The proenzyme form of DPAP1 was found to accumulate in the parasitophorous vacuole of mature parasites. This observation suggests a trafficking route for DPAP1 through the parasitophorous vacuole to the food vacuole.

As the etiologic agent of severe malaria in humans, the Apicomplexan parasite *Plasmodium falciparum* is one of the leading causes of morbidity and mortality among infectious diseases. *P. falciparum* takes its greatest toll in sub-Saharan Africa, where at least 90% of the 1–2 million annual deaths due to falciparum malaria occur (1). The spread of parasites resistant to effective and affordable antimalarials such as chloroquine has led to a worsening of the situation. With a vaccine still many years away, the development of novel antimalarial chemotherapeutic agents that are efficacious against multidrug-resistant parasites remains an urgent priority.

Although *P. falciparum* has a complex life cycle that requires both mosquito and human hosts, the pathology caused by this parasite occurs while it reproduces asexually in human erythrocytes. During its intraerythrocytic development the parasite internalizes and degrades up to 75% of erythrocyte hemoglobin. Amino acids derived from hemoglobin catabolism are incorporated into plasmodial proteins (3), and parasites can rely on hemoglobin catabolism to supply sufficient quantities of all amino acids except those five that are rare in or absent from hemoglobin (4, 5). Based on these observations, a supply of amino acids for parasite protein synthesis has been considered the primary benefit of hemoglobin catabolism; however, recent studies suggest that this may not be the only benefit. A quantitative analysis of the incorporation of hemoglobin-derived amino acids into *P. falciparum* proteins has revealed that the parasite utilizes less than one-fifth of the amino acids made available through hemoglobin catabolism (6). This observation coupled with modeling of erythrocyte volume during intraerythrocytic growth has led to the suggestion that hemoglobin uptake and degradation may also be important in preventing hemolysis of *P. falciparum*-infected erythrocytes (7, 8).

In *P. falciparum*, hemoglobin is degraded in a single, acidic organelle known as the food vacuole or digestive vacuole. The initial stages of hemoglobin catabolism have been well characterized and involve a diverse set of endopeptidases. The initial proteolytic cleavage occurs at the hinge region of the α-globin chain and is mediated by the aspartic proteases plasmepsins I, II, and IV (9, 10). Further degradation of the globin chains is achieved through the action of plasmepsins I, II, and IV (10–12), histo-aspartic protease (11), and three cysteine proteases, falcipain-2, -2′, and -3 (13–15). The metalloprotease falcilysin converts short globin polypeptides into oligopeptides consisting of 5–10 amino acids (16). Inhibitors of both plasmepsins and falcipains block hemoglobin degradation and kill *P. falciparum* (5, 17). Interruption of hemoglobin catabolism is, therefore, an attractive strategy in the search for novel antimalarial chemotherapeutics.

The means by which oligopeptides are degraded to amino acids remain unclear. In a study addressing this question, Kolakovich et al. (50) asked whether amino acids can be generated from hemoglobin by food vacuole extract in vitro. The lack of detectable exopeptidase activity in this experiment suggested that hemoglobin catabolism may not occur to completion in the food vacuole. A model for hemoglobin degradation was proposed that envisioned export of hemoglobin-derived oligopeptides from the food vacuole to the cytoplasm for terminal degradation by neutral aminopeptidases. This model has been supported by the identification of cytosolic aminopeptidase activity in *P. falciparum* that can partially degrade hemoglobin peptides (18) and the description of a cytosolic zinc aminopeptidase with broad substrate specificity that is enriched at the cytosolic face of the food vacuole (19). However, direct evidence...
for the transport of oligopeptides out of the food vacuole has not yet been reported.

To further our understanding of protein degradation in the food vacuole and to identify potential novel drug targets, we have mined the P. falciparum genome sequence data base for homologs of exopeptidases that function in acidic organelles. We identified a plasmoidal homolog of mammalian type I dipeptidyl aminopeptidase (pDAPA1) lysosomal exopeptidase that sequentially cleaves dipeptides from the N terminus of its oligopeptide substrate (20). Cathepsin C also plays a critical role in the activation of several serine proteases of immune effector cells (21–23). Given the role of cathepsin C in lysosomal protein degradation, we considered the possibility that the plasmoidal enzyme might have a similar function in the food vacuole. To investigate this we undertook a detailed characterization of the P. falciparum dipeptidyl aminopeptidase, examining its enzymatic activity, its importance for axenial replication, and its location in the parasite.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture and Transfection—**P. falciparum clones 3D7 and HB3 were cultured in human O+ erythrocytes in RPMI 1640 supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and either 5 μl/100 ml Albumax (In Vitrogen; for routine growth) or 10% heat-inactivated human O− serum (for transfaction and cycling) as previously described (24). Cultures were synchronized by sorbitol treatment (25).

A dipeptidyl aminopeptidase type I (pDAPA1) truncation plasmid (pFA5KO) was PCR amplified by PCR amplification of bases 928–1857 of the pDAPA1 open reading frame with primers 5′-AGCATCGTGAATCTGGATGATTCTAATTCCTTTGCATTTTTTC and introduced into the XhoI/AvrII sites of pPM2GT (29) to yield pFA5GT. After reading frame (omitting the stop codon) was PCR amplified from primers 5′-AGCATCGTGAATCTGGATGATTCTAATTCCTTTGCATTTTTTC and introduced into the XhoI/AvrII sites of pPM2GT (29) to yield pFA5GT. After electroporation of 3D7 rings with 100 μg of pFA5GT, parasites containing the sequence acetyl-TKKLDRKYLNNFDD (Research Genetics) and were affinity-purified using Sepharose-coupled peptide.

Immunoblotting was carried out with monoclonal anti-DPAPA1 antibody 244 (1:200; Figs. 2C and 5), affinity-purified rabbit anti-DPAPA1 antibody 1502 (1:500; Fig. 2C), affinity-purified rabbit anti-GFP antibody 53D3 (1:10,000, Abcam), affinity-purified rabbit anti-BIP (1:50), and mouse anti-P47/serine-rich antigen (SERA; 0.1 μg/ml (31)). For immunoprecipitation, synthesized 3D7 or F9 trophozoites at 2% hematocrit and 10–15% parasitemia were labeled for 3 h in RPMI 1640 lacking cysteine and methionine (Sigma) supplemented as described under "Parasite Culture and Transfection" under "Experimental Procedures" and containing 90 μM 3′S)methionine and -cysteine (Exprea®S®S, PerkinElmer Life Sciences). Labeled intact erythrocytes were washed three times with cold phosphate-buffered saline, resuspended in phosphate-buffered saline, and lysed by the addition of IP buffer (1× IP buffer is 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 6 mM EDTA, 1% Triton X-105, 0.5% deoxycholate, and the following protease inhibitors: 1 μM pepstatin, 1 μM aminopeptidase N, 1 μM aminopeptidase V, 1 μM N-(trans-epoxysuccinyl)-L-leucine-4-guanidinobutyramide, and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride). After removal of insoluble material, labeled proteins were immunoprecipitated at 4°C with anti-DPAPA1 antibody 244 (1:20) or Living Colors full-length Aplysia polyvalent anti-GFP antibody (1:1000, BD Biosciences). Immune complexes were dissociated by boiling in reducing SDS-PAGE sample buffer, and proteins were analyzed by 12% SDS-PAGE and autoradiography.

**DAPA1 Purification and Assay—**DAPA1 was immunoprecipitated for 1 h at 4°C from trophozoite lysate with monoclonal anti-DPAPA1 antibody 244 and protein A-Sepharose beads (Amersham Biosciences) in 50 mM bis-tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1 μg pepstatin (binding buffer). The beads were washed 3 times in binding buffer, once in wash buffer (10 mM bis-tris-HCl, pH 7.0, 150 mM NaCl) and were then added to assay mixtures containing 100 μM sodium acetate, pH 5.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 100 μM β-naphthylamide substrate (Bachem). Assays were mixed continuously for 1 h at 37°C, with no detectable dipeptidase activity if either the antiserum or control rabbit serum was added to the assay mixture. Fluorescence was determined from parallel samples lacking enzyme. In control experiments, no dipeptidase activity was detected if either the anti-DPAPA1 hybridoma supernatant containing antibodies to the aspartic protease plasminogen V (11) was used.

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phosphate buffers adjusted to pH values between 4.5 and 7.5 for 30 min at 37°C. Fluorescence and Immunoelectron Microscopy—Fluorescence and phase contrast images were collected with an Axioskop epifluorescence microscope equipped with a Plan-Neofluar Ph3 100x/1.3NA objective, an Axiocam CCD camera, and Axiovision 3.1 software (Carl Zeiss Microimaging). Parasites were mounted directly from culture and observed at ambient temperature. Parasite DNA was visualized by adding 5μM Hoechst 33342 (Molecular Probes) immediately before mounting. Immunoelectron microscopy was performed on parasites fixed with 4% paraformaldehyde, 0.1% glutaraldehyde and immunogold-labeled with anti-GFP antibody 6556 (1:1000, Abcam) or anti-DPAP1 monoclonal antibody 244 (undiluted hybridoma supernatant) as previously described (29).

Parasite Fractionation—Erythrocytes infected with schizont stage parasites were enriched to 65–75% parasitemia on a 65% Percoll column (33). Saponin (Sigma) fractionation was carried out at a final concentration of 0.05% essentially as previously described (34). Fractionation with 3μg/ml tetanolysin (35) was performed in a similar manner to that previously described for streptolysin O (36).

RESULTS

A Dipeptidyl Aminopeptidase Is Active in Blood Stage P. falciparum—We have identified three open reading frames encoding putative DPAP homologs (loci PF11_0174, PFL2290w, and PFD0230c) in a BLAST search of the P. falciparum genome sequence (37). A reverse transcription-PCR analysis of mRNA levels of the three homologs in intraerythrocytic parasites (data not shown) indicated that sequence PF11_0174 had a broad expression profile that included the stage during which the parasite was most actively degrading hemoglobin. In contrast, expression of the other two homologs was minimal in trophozoites and peaked in schizonts. From this analysis, PF11_0174 seemed to be the best candidate for a role in hemoglobin degradation and was, therefore, studied in greater detail. This sequence will be referred to here as P. falciparum DPAP homolog 1 or DPAP1.

The predicted amino acid sequence of DPAP1 shows significant homology to that of the human DPAP cathepsin C (Fig. 1). Identity/similarity over the exclusion domain, the prodomain, and the papain-like catalytic region is 26/40%, 16/32% and 38/54%, respectively (excluding gap positions). Over the entire sequence the two proteins share 30% identity and 45% similarity (excluding gap positions). Several residues known to be important for peptidase activity are conserved in DPAP1; they are Asp-28, which in cathepsin C is the N-terminal residue of the exclusion domain and blocks the substrate binding cleft beyond the P2 site (38, 39), and Cys-398, His-624, and Asn-648, which form the cysteine protease catalytic triad (40). A tyrosine residue that binds a chloride ion in the crystal structures of rat and human cathepsin C (38, 39) is also conserved (Tyr-549). The presence of a putative signal sequence at the N terminus of DPAP1 suggests that this protein transits the P. falciparum secretory system.

To determine whether DPAP1 exhibits dipeptidyl aminopeptidase activity, the protein was immunoprecipitated from soluble trophozoite extract with an anti-DPAP1 monoclonal antibody 244 (undiluted hybridoma supernatant) as previously described (29).

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DPAP1 cleaves unblocked dipeptide substrates, except for dipeptides having a basic residue in the first position or proline in the second position. To confirm that this activity was due to DPAP1 and not one of the other two DPAP homologs, soluble trophozoite extract was fractionated over consecutive hydrophobic interaction, anion exchange, and gel filtration columns. DPAP1 was observed to comigrate with dipeptidase activity (Fig. 2C and data not shown). In the pH range 4.5 to 7.5, DPAP1 activity was greatest at pH 5.5–6.0, with the activity at pH 7.5 only 5% of the value at pH 5.5 (data not shown). This result is consistent with a role for DPAP1 in the acidic lumen of the food vacuole.

The production of mature, active cathepsin C requires endoproteolytic processing of the proenzyme to remove the internal proregion (44). To determine whether DPAP1 is similarly processed, DPAP1 polypeptides were immunoprecipitated from 35S-labeled trophozoites (Fig. 2B). A high molecular mass species was observed, the size of which is similar to that predicted for the proenzyme (proDPAP1) after signal peptide cleavage (77.4 kDa). In addition, three polypeptides with apparent molecular masses of 18–21 kDa were immunoprecipitated. By analogy with cathepsin C, these polypeptides were presumed to correspond to mature, active DPAP1 generated by excision of the prodomain and a single cleavage within the catalytic region (44). To confirm this, DPAP1 activity was partially purified from trophozoite extract by column chromatography and was subjected to immunoblotting with two DPAP1-specific antibodies (Fig. 2C). Only low molecular weight polypeptides and not the 77-kDa proenzyme were detected in the preparation of active DPAP1. Based on the known proteolytic processing sites in human cathepsin C (Fig. 1) and the sizes of the mature DPAP1 polypeptides (Fig. 2B), a model for the proteolytic activation of DPAP1 is proposed in Fig. 2D.

**DPAP1 Is Important for Asexual Parasite Proliferation**—To assess the importance of DPAP1 during the intraerythrocytic cycle, we attempted to disrupt the chromosomal copy of the DPAP1 gene by single-crossover recombination with a plasmid carrying a truncated segment of the coding sequence. The resulting protein would lack the last 81 residues, including the histidine residue of the catalytic dyad (Fig. 3A, i). As a control, integration of a 3′ replacement plasmid that carries the complete 3′ end of the coding sequence (and, therefore, does not truncate the protein) was attempted in parallel (Fig. 3A, ii). Parasites stably transfected with each plasmid were subjected to drug cycling to promote integration of the plasmids at the DPAP1 gene locus. A parasite population containing the integrated 3′ replacement plasmid was observed after a single drug cycle (Fig. 3B). In contrast, no detectable integration of the truncation plasmid was observed after two drug cycles (Fig. 3B). The inability to force integration of the truncation plasmid suggests that inactivation of DPAP1 is lethal or highly deleterious to blood stage parasites.

**DPAP1 Resides in the Food Vacuole**—To localize DPAP1 in asexual blood stage parasites, we created a cloned line of parasites (designated F9) in which a sequence coding for a linker followed by GFP was integrated at the 3′ end of the coding region of the endogenous DPAP1 gene (Fig. 3, A, iii, and C). Immunoprecipitation of DPAP1 and GFP from F9 trophozoite extracts revealed that proDPAP1 was synthesized with the GFP tag, but that GFP was lost from mature DPAP1 (Fig. 3C). This loss of GFP was indicated by the close similarity of the sizes of mature DPAP1 polypeptides from F9 (Fig. 3D, anti-DPAP1 (α-DPAP1) lane) and wild-type 3D7 (Fig. 2B) parasites and by the appearance of a 27-kDa GFP species (Fig. 3D, anti-GFP (α-GFP) lane). These data also demonstrated that the GFP tag did not impede proper maturation of DPAP1. Because DPAP1 is an exopeptidase, cleavage of the linker between DPAP1 and GFP is probably mediated in trans by an endopeptidase.

The distribution of GFP in live *P. falciparum*-infected erythrocytes was assessed by epifluorescence microscopy. The asex-
The parasite during invasion and remains intact until shortly before daughter merozoite egress (45, 46). In F9 schizonts undergoing nuclear division, PV fluorescence was evident as an outline around the periphery of the parasite (Fig. 4, B and C), whereas in mature segmented schizonts, the fluorescence could be seen around daughter merozoites (Fig. 4D). These schizont PV fluorescence patterns are similar to those reported previously in parasites expressing chimeric GFP proteins that target to the PV (47–49).

The presence of GFP in the PV raised the intriguing possibility that DPAP1 has a specific role in this compartment. To test whether proDPAP1 is processed to the catalytically active mature form in the PV, F9 schizonts were fractionated by treatment with tetanolysin, a protein that forms pores in the erythrocyte membrane, and with saponin, a detergent that permeabilizes both the erythrocyte and PV membranes (Fig. 5A). ProDPAP1-GFP was found in the saponin but not the tetanolysin supernatant, which indicates that this species is present in the PV but not in the erythrocyte cytosol (Fig. 5B). In contrast, neither GFP nor mature DPAP1 was detected in the saponin or tetanolysin supernatants. These species were found exclusively in the parasite pellets, a result consistent with residence within the food vacuole. These fractionation data indicate that proDPAP1 processing and GFP cleavage do not occur in the PV. To confirm that PV accumulation of DPAP1 is not an artifact of the GFP tag, wild-type 3D7 schizonts were fractionated with tetanolysin and saponin (Fig. 5C). Results
were similar to those obtained with F9 parasites. In both experiments, the PV-resident 120-kDa SERA was used as an indicator of PV permeabilization, and the endoplasmic reticulum marker BiP was employed as a control for the integrity of the parasite.

In trophozoites and to a lesser degree in schizonts, bright GFP fluorescence was observed in mobile punctate spots. These spots became evident in very young trophozoites (Fig. 6A) and appeared to be outside the parasite yet were often very close to it with movement restricted to the outer parasite periphery. Occasionally, a fluorescent spot could be observed at a significant distance from the parasite. The average number of fluorescent spots per trophozoite was 3.6 \pm 1.8 (n = 75). Treatment of infected erythrocytes with saponin did not result in loss of GFP from these structures (data not shown), which suggests that they are not contiguous with the parasitophorous vacuole/FIG. 4. Localization of DPAP1 to the food vacuole and the parasitophorous vacuole. A–D, fluorescence microscopy of live parasites expressing DPAP1-GFP. A, trophozoite. B, ~2N schizont. C, ~8N schizont. D, 8N segmented schizont. In the center column fluorescence from GFP is green and that from nuclear stain Hoechst 33342 is red. The food vacuole is indicated with arrowheads in the GFP panels of A–C, and the parasitophorous vacuole is indicated with arrows in B and C. Fluorescent spots in B and C may be vesicular structures involved in transport of DPAP1-GFP to the food vacuole. Bar, 2 \mu m. E, immunogold labeling of an F9 parasite with anti-DPAP1 antibody. fv, food vacuole; er, endoplasmic reticulum; n, nucleus. Bar, 400 nm.

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Fig. 6. DPAP1-GFP accumulates in vesicles outside the parasite. A, fluorescence image of a young F9 trophozoite showing GFP-containing foci (left) that in a fluorescence-phase contrast overlay (right) appear to lie outside the parasite. Bar, 2 μm. B and C, immunogold labeling of F9 parasites with either anti-GFP (B) or anti-DPAP1 antibody (C). Significant accumulations of gold label are observed in vesicles closely apposed to the parasitophorous vacuole membrane (thick arrows). fv, food vacuole; ppm, parasite plasma membrane; pvm, parasitophorous vacuole membrane; em, erythrocyte membrane; n, nucleus. Bar, 200 nm.

DISCUSSION

Although the initial stages of hemoglobin degradation in the P. falciparum food vacuole have been intensely studied, the final steps in the conversion of globin peptides to amino acids have received scant attention. Oligopeptides of 5–10 amino acids were thought to be the end products of hemoglobin catabolism within the acidic food vacuole based largely on an in vitro analysis of hemoglobin degradation by food vacuole extract that failed to detect exopeptidase activity (50). Our results presented here strongly suggest that exopeptidase activity does indeed reside in the food vacuole. P. falciparum DPAP1, a cathepsin C ortholog, both possesses acidic dipeptidyl aminopeptidase activity and is located in the food vacuole at the time of hemoglobin degradation. The probable role of DPAP1 is the release of dipeptides from the N termini of oligopeptides produced through the concerted action of food vacuole aspartyl-, cysteinyl- and metallo-endoproteases (Fig. 7). The failure to detect DPAP1 activity in vitro in food vacuole extract may derive from the lack of exogenously added reducing agent (50); a reducing environment is required to prevent oxidative inactivation of DPAP1 in vitro (data not shown).

How the dipeptides generated by DPAP1 are hydrolyzed to amino acids remains unclear. They may be actively transported out of the food vacuole in a similar fashion to that observed with lysosomes (51) for cleavage by cytosolic neutral aminopeptidases. Alternatively, as-of-yet undiscovered acidic aminopeptidase activity may contribute to the hydrolysis of dipeptides within the food vacuole. Indeed, eight aminopeptidase homologs have been identified in the P. falciparum genome sequence (52), and only one these, a cytosolic M1 family zinc neutral aminopeptidase, has been characterized at the protein level (19, 53).

The inability to inactivate DPAP1 in blood stage parasites by truncation of the coding sequence provides evidence that its loss is lethal or highly deleterious to parasite proliferation. The importance of DPAP1 for intraerythrocytic growth makes this enzyme an attractive target for the development of novel antimalarial protease inhibitors that kill the parasite by blocking the later stages of hemoglobin degradation. This approach could complement current efforts to disrupt the early stages of hemoglobin degradation by inhibiting plasmepsins and falcipains (54). Although the two other cathepsin C homologs identified in the P. falciparum genome are expressed during the intraerythrocytic cycle (55, 56), they are apparently unable to compensate for the loss of DPAP1. If either of these homologs resides in the food vacuole, it may possess a complementary substrate specificity to that of DPAP1; alternately, these proteins may have functions completely unrelated to hemoglobin degradation. The presence of at least two cathepsin C homologs in the genome of the intestinal parasite Cryptosporidium parvum (57) provides a hint that these enzymes have roles to play in Apicomplexan biology outside of hemoglobin catabolism.

The presence of proDPAP1 but not mature DPAP1 in the PV of schizonts suggests a trafficking route for proDPAP1 to the food vacuole. In this model proDPAP1 would traffic to the PV via the parasite default secretory pathway (47–49). From there, proDPAP1 could be transported to the food vacuole in

HEMOGLOBIN

Plasmepsins I, II, IV

GLOBIN POLYPEPTIDES

Plasmepsins I, II, IV Falcipain-2, -3 Hist-aspartic protease

OLIGOPEPTIDES (10-20 RESIDUES)

Falcylisin

OLIGOPEPTIDES (5-10 RESIDUES)

DPAP1

Dipeptides

Fig. 7. Peptidases contributing to the degradation of hemoglobin inside the P. falciparum food vacuole. Endopeptidases are indicated in italics, and the sole known exopeptidase, DPAP1, is indicated in bold italics.
double-membrane transport vesicles that bud from the cytosome, the site of hemoglobin endocytosis. The transport vesicles develop from an invagination of the PV and parasite plasma membranes (58); the space between these membranes is contiguous with the PV lumen and could be utilized to transport soluble proteins from the PV to the food vacuole. Transport of the membrane-anchored proplasmepsins I and II via cytosome-derived transport vesicles has been previously described (5, 29). The plausibility of this model for DPAP1 is supported by the observation that a fraction of the GFP in the PV of parasites expressing signal sequence-GFP fusions ends up in the food vacuole (47–49). Based on the steady-state amounts of GFP in the PV and food vacuole in these studies, this mode of transport appears relatively inefficient; therefore, DPAP1 may possess specific targeting signals directing it to the cytostome and, hence, to the food vacuole. The observed accumulation of proDPAP1 in the PV of schizonts but not trophozoites may simply derive from the reduction in cytostomal activity as the parasite matures. In the absence of a route out of the PV in schizonts, any newly synthesized DPAP1 would accumulate in this compartment.

In parasites expressing DPAP1-GFP, we observed mobile fluorescent spots outside the parasite. These spots are unlikely to be subdomains of the PV or tubovesicular network, as GFP was not released from them by saponin treatment. At ultrastructural resolution, these spots appeared to be vesicles in close apposition to the PV membrane. The only other structure to our knowledge that exhibits a punctate distribution in the erythrocyte cytosome is Maurer’s clefts, which are implicated in trafficking of proteins to the erythrocyte membrane (59). The vesicles that we observe are not Maurer’s clefts. At the ultrastructural level, Maurer’s clefts appear as flattened, elongated cisternae near the erythrocyte membrane (60, 61) that may be part of a continuous membrane network that includes elements of the tubovesicular network (62). In contrast, the DPAP1-containing structures are round or irregular oval in shape and are in close proximity to the parasite. Moreover, Maurer’s clefts are much more numerous than the three to four fluorescent spots that we typically observe in trophozoites (see for example Fig. 1 in Ref. 61). These intriguing structures will be the subject of further study.

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