Roles for Two Aminopeptidases in Vacuolar Hemoglobin Catabolism in Plasmodium falciparum*

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During the erythrocytic stage of its life cycle, the human malaria parasite Plasmodium falciparum catabolizes large quantities of host-cell hemoglobin in an acidic organelle, the food vacuole. A current model for the catabolism of globin-derived oligopeptides invokes peptide transport out of the food vacuole followed by hydrolysis to amino acids by cytosolic aminopeptidases. To test this model, we have examined the roles of four parasite aminopeptidases during the erythrocytic cycle. Localization of tagged aminopeptidases, coupled with biochemical analysis of enriched food vacuoles, revealed the presence of amino acid-generating pathways in the food vacuole as well as the cytosol. Based on the localization data and in vitro assays, we propose a specific role for one of the plasmodial enzymes, aminopeptidase P, in the catabolism of proline-containing peptides in both the vacuole and the cytosol. We establish an apparent requirement for three of the four aminopeptidases (including the two food vacuole enzymes) for efficient parasite proliferation. To gain insight into the impact of aminopeptidase inhibition on parasite development, we examined the effect of the presence of amino acids in the culture medium of the parasite on the toxicity of the aminopeptidase inhibitor bestatin. The ability of bestatin to block parasite replication was only slightly affected when 19 of 20 amino acids were withdrawn from the medium, indicating that exogenous amino acids cannot compensate for the loss of aminopeptidase activity. Together, these results support the development of aminopeptidase inhibitors as novel chemotherapeutics directed against malaria.

With an estimated 2 million deaths annually to its name (1), the human malaria parasite Plasmodium falciparum continues to present an enormous public health challenge. Clinical manifestations of infection appear as the parasite replicates within host erythrocytes. During this replication cycle, the parasite endocytoses and catabolizes large amounts (up to 75%) of host hemoglobin to constituent amino acids (2, 3). This process makes available large quantities of free amino acids for parasite protein synthesis (4) and may also modulate the osmotic environment of the host cell (5) and/or create space inside the red cell for parasite growth (6). Amino acids derived from hemoglobin catabolism are also important for the uptake of isoleucine from the extracellular environment (7). As hemoglobin catabolism is essential for parasite replication and is currently a major focus of anti-malarial drug development efforts, a comprehensive understanding of the biochemistry of this pathway is an urgent priority.

Hemoglobin catabolism occurs in an acidic, degradative organelle termed the food vacuole (FV)2 or digestive vacuole. In the lumen of the FV, endopeptidases of diverse catalytic mechanism and specificity (plasmspepsin (PM) I, II, and IV, histo-aspartic protease, falcipain-2, -2', and -3, and falcilysin) contribute to the hydrolysis of the α- and β-globin chains to oligopeptides (8, 9). These oligopeptides are further hydrolyzed to dipeptides by the FV exopeptidase dipeptidyl aminopeptidase 1 (10). The fate of these di- and oligopeptides is unclear. A widely cited model holds that the terminal stages of hemoglobin degradation occur in the cytosol, with peptides exported from the FV for hydrolysis by cytosolic aminopeptidases (11, 12). This model is based largely on the apparent lack of aminopeptidase (AP) activity in extracts of enriched FVs (12), on the localization of two P. falciparum APs to the cytosol (13–15), and on the apparent absence of carboxypeptidase homologs from the annotated parasite genome sequence (16). Support for this model is weakened, however, by the absence of experimental evidence for peptide export from the FV, and by the presence of uncharacterized AP homologs in the P. falciparum genome sequence that could play a role in vacuolar peptide catabolism.

To test this model, we have examined the roles of four P. falciparum AP homologs. These four enzymes were selected because, to the best of our knowledge at the time of this study, they constituted the entire repertoire of AP activities encoded by the P. falciparum genome, excluding the housekeeping methionine aminopeptidases. To gain insight into the functions of these four APs, we have determined the subcellular distribution of each. We have attempted to inactivate each AP by disrupting its coding sequence to assess their importance for...
intraerythrocytic growth and to determine their utility as potential drug targets. To further probe the roles of APs in peptide catabolism and parasite growth, the effect of exogenous amino acids on the toxicity of the AP inhibitor bestatin was examined.

EXPERIMENTAL PROCEDURES

**Generation of Constructs**—The green fluorescent protein allele gfpmut2 (17) was converted to the yellow fluorescent protein (YFP) allele Citrine (18) by introduction of the mutations A65G, Q69M, and T203Y using the QuikChange mutagenesis kit (Stratagene). The mutation A206K was also introduced to eliminate the weak tendency of GFP to dimerize (19). The Citrine sequence, preceded by the tobacco etch virus protease cleavage site (ENLYFQS) and followed by the Softag1 epitope tag GSLAELNAGLGG (20), was introduced into the AvrII/NotI sites of the plasmid pPM2GT (21) to produce pPM2CIT2. To create plasmids for the generation of chromosomal AP-YFP fusions, 3′-1-kb fragments of PfAPP, PfDAP, PfA-M1, and PfLAP coding sequence up to but not including the stop codon were PCR-amplified from *P. falciparum* clone 3D7 genomic DNA and cloned into the XhoI/AvrII sites of pPM2CIT2. TA tag constructs were produced by excising the AvrII/NotI YFP fragment from the AP-YFP plasmids and cloning in-frame a sequence encoding GGGYPYDVPDYA (HA tag underlined) into the same sites. For gene disruption constructs, a kilobase of sequence from the 5′ end of each AP coding sequence was cloned into the XhoI/AvrII sites of pPM2CIT2. Sequences of primers used for PCR amplification of AP sequences are provided in supplemental Table S1.

**Parasite Culture and Transfection**—*P. falciparum* clone 3D7 was grown in human O+ erythrocytes in RPMI medium supplemented as described previously (21). RPMI containing 148 μM isoleucine as the only amino acid (I medium) was prepared as described (22). Cultures were synchronized by sorbitol treatment (23). Parasites were transfected with 75–100 μg of plasmid DNA using low voltage electroporation conditions (24), and 10 nM WR99210 was added after 48 h. Resistant parasites appearing after 18–24 days were subjected to drug cycling whereby WR99210 was removed from the media for 21 days and then applied until resistant parasites reappeared. After two drug cycles, populations of parasites expressing PfDAP-YFP, PfAPP-YFP, and PfLAP-YFP were obtained. All parasites in each population appeared to possess a chromosomal AP-YFP chimera, as nonfluorescent parasites were no longer observed. In contrast, after two drug cycles only a low percentage of parasites expressed PfA-M1-YFP, as determined microscopically; this necessitated the isolation of clonal fluorescent parasite lines by limiting dilution. Clone F11 was used for this study. Parasites stably transfected with gene disruption episomes were cycled off/on drug two (PfDAP) or three times (PfAPP, PfLAP, and PfA-M1). Clonal parasite lines containing a disrupted PfDAP gene were obtained by limiting dilution; clone C2 was used for this study.

**Southern Blot Analysis of Transfected Parasites**—To detect targeted integration of the episome in parasite lines that were expressing YFP or HA fusions or were transfected with gene disruption constructs, genomic DNA was isolated from saponin-treated parasites using the QiaAmp DNA blood mini kit (Qiagen), digested with one or two restriction enzymes (enzymes used for YFP fusion and gene disruption lines are indicated in supplemental Figs. S1 and S8, respectively), and resolved on a 0.6% agarose gel. After transfer to a Nytran+ membrane (GE Biosciences) and blocking, AP loci were detected using probes complementary to the episomal AP targeting sequences. Probe labeling and detection were carried out using the AlkPhos direct labeling kit (GE Biosciences).

**Fluorescence Microscopy**—For live imaging of parasites expressing AP-YFP fusions, cultures were mounted under a coverslip after addition of the vital nuclear stain Hoechst 33342 (5 μM). Images were collected on a Zeiss AxioImager equipped with an MRm Axiocam digital camera using a 100×/1.4NA objective lens. For HA tag localization, parasites were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde, permeabilized, and blocked, as described previously (25), and then incubated with an affinity-purified rabbit anti-HA antibody (Invitrogen) followed by an Alexa 594-conjugated anti-rabbit secondary antibody (Invitrogen). Cells were then allowed to settle onto polyethyleneimine-coated coverslips and were mounted with ProLong Gold containing the nuclear stain 4′,6-diamidino-2-phenylindole (Invitrogen). Images were converted to TIF files, and contrast was adjusted using Adobe Photoshop CS2.

**Trophozoite and FV Isolation and Preparation of Extracts**—Trophozoites were isolated from infected erythrocyte cultures by treating with 1 mg/ml saponin in Dulbecco’s phosphate-buffered saline (PBS) for 10 min on ice. Parasites were recovered by centrifugation at 1940 × g for 10 min at 4 °C, washed once with cold PBS, and then stored at −80 °C. Extracts used in Fig. 5C were prepared by resuspending parasite pellets in 500 μl of 25 mM sodium MOPS, pH 7.0, 100 mM NaCl and sonicating three times for 10 s. Soluble extracts were generated by centrifuging the crude lysate at 100,000 × g for 1 h at 4 °C. For normalization of enzyme rate data, the parasitemia of infected erythrocyte cultures was determined from a Giemsa-stained thin smear and used to calculate the number of parasites in the extract.

To prepare extracts for immunoblotting, synchronized trophozoite and schizont parasites were isolated by saponin treatment as described in the above paragraph and stored at −80 °C. Frozen parasite pellets were resuspended in PBS containing the following protease inhibitors: 10 μM pepstatin, 10 μM N-(trans-epoxysuccinyl)-l-leucine 4-guanidinobutylamide (E-64), 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 1 mM sodium EDTA. Inclusion of these inhibitors was required to prevent artifactual proteolysis of AP-YFP fusions during sample preparation. Reducing SDS-PAGE sample buffer was added, and the sample was mixed and immediately placed in a boiling water bath for 3 min. Insoluble material was removed by centrifugation at 12,000 × g for 2 min.

Food vacuoles were prepared from a parasite line expressing PM II–GFP (21) as described previously (26). Briefly, ∼5 × 10⁹ saponin-treated trophozoites were washed twice with cold PBS and resuspended in 500 μl of cold water that had been adjusted to pH 4.5 with HCl. Resuspended parasites were triturated four times through a 27-gauge needle. After centrifugation at
TABLE 1

**P. falciparum** aminopeptidases examined in this study

The nearest homolog is taken from Ref. 16. Reported specificities are listed for PfA-M1 (13) and PFLAP (15); those for PfAPP and PfDAP are inferred from homology. The P1 position is the first residue of the substrate. X represents any amino acid. The presence of a signal peptide was determined by inspection of the annotated protein sequence. Cyt, cytosol; Nuc, nucleus.

| Name    | Gene ID | Nearest homolog      | Specificity  | Signal peptide | Localization |
|---------|---------|----------------------|--------------|----------------|-------------|
| PFAPP   | PF14_0517| Aminopeptidase P     | X-Pro        | Yes            | FV, Cyt     |
| PFA-M1  | MAL13P1.56| Aminopeptidase N     | Broad P1     | Yes            | FV, Nuc     |
| PFLAP   | PF14_0439| Leucyl aminopeptidase| Hydrophobic P1 | No             | Cyt         |
| PFAPP   | PF1570c | Aspartyl aminopeptidase| Asp/Glu-X     | No             | Cyt         |

Enrichment of DPAP1 and Ala-Pro-Ala-βNA Assays—Approximately 10⁶ trophozoite-stage parasites were suspended in 50 mM BisTris-HCl, pH 6.0, and lysed by sonication. The extract was clarified by centrifugation at 100,000 × g for 1 h at 4 °C. Supernatant was loaded onto a MonoQ column equilibrated in 50 mM BisTris-HCl, pH 6.0, and proteins were eluted with a linear gradient from 0 to 1 M NaCl. Fractions containing DPAP1 were identified using the substrate PR-AMC as described under “Enzyme Assays.” Assays for Ala-Pro-Ala-βNA hydrolysis contained either clarified FV lysate, prepared as described above, or 5 μl of a MonoQ fraction enriched in DPAP1 supplemented with 0, 0.3, 1, or 3 μg of purified recombinant PfAP1 in 50 mM sodium MOPS, pH 5.5, 2 mM dithiothreitol, 30 mM NaCl, and 500 μM Ala-Pro-Ala-βNA (Bachem). Assays of FV extract also contained 0.1% Triton X-100. When inhibitors were included in the assay, concentrations were as follows: 1 μM E-64, 10 μM bestatin, 1 μM pepstatin, or 1 μM Pro-Arg-fluoromethyl ketone (PR-FMK). All inhibitors were obtained from commercial sources except PR-FMK, which was custom-synthesized (Enzyme Systems Products).

Bestatin Treatment—*P. falciparum* 3D7 parasites were cultured for at least three generations in I medium. Aliquots of a synchronous ring-stage parasite culture were centrifuged at 860 × g for 3 min, and infected cell pellets were resuspended in either fresh I medium or in regular RPMI medium containing all 20 amino acids. Bestatin was added to 200-μl aliquots of cultures in 96-well flat-bottom culture dishes to give a concentration range of 0.03–30 μM. A separate aliquot of culture was resuspended in RPMI medium lacking all amino acids to induce amino acid starvation. All assays were carried out in triplicate. Parasite cultures were incubated for 65 h and then fixed with 0.1% glutaraldehyde in PBS. After permeabilization of cells with 0.25% Triton X-100 in PBS and staining of DNA with 400 nM YOYO-1 (Invitrogen) in PBS, parasite fluorescence was determined on a Coulter Epics XL MCL flow cytometer equipped with a 488 nm laser. Flow cytometry histogram overlays, parameters, and mean fluorescence intensities were generated using FlowJo version 8.3 (Treestar).

RESULTS

**Aminopeptidases in the *P. falciparum* Genome**—The four APs studied here were identified through molecular biological (31) and bioinformatic (16) approaches. The designated names and nearest homologs of these sequences are listed in Table 1. One of them, aminopeptidase N (PFA-M1), has broad specificity at the substrate P1 position (13), whereas that of leucyl aminopeptidase in cold uptake buffer (25 mM HEPES, 25 mM NaHCO₃, 5 mM sodium phosphate, 100 mM KCl, 10 mM NaCl, 2 mM MgSO₄, pH 7.4). DNase I was added (5 μl of a 5 mg/ml solution), and the mixture was incubated for 3 min at 37 °C. After centrifugation at 18,000 × g for 2 min, the pellet was resuspended in 100 μl of cold uptake buffer, added to 1.3 ml of 42% Percoll, 0.25 M sucrose, 1 mM MgCl₂, pH 7.4, triturated twice as described above, and centrifuged at 18,000 × g for 10 min at 4 °C. The material at the bottom of the tube was washed once with uptake buffer, resuspended in 100 μl of uptake buffer, and then subjected to a second Percoll enrichment step. The second enrichment, which was carried out exactly as the first, reduced contamination with unlysed trophozoites. The presence of intact, GFP-containing FVs was confirmed microscopically. Isolated vacuoles were stored at −80 °C. Extracts of FVs and trophozoites (for Fig. 3) were prepared by suspension in 25 mM sodium MOPS, pH 7.0, 100 mM NaCl, 0.1% Triton X-100, and insoluble material was removed by centrifugation at 100,000 × g for 30 min at 4 °C.

Immunoblotting—Parasite extracts were resolved on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with affinity-purified anti-GFP antibody 6556 (Abcam, 1:10,000 dilution) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Biosciences). Chemiluminescent signal was developed with the ECL kit (GE Biosciences). Blots were stripped and reprobed with rabbit anti-BiP (1:10,000 (27)) to assess relative loading levels.

Enzyme Assays—Peptidase assays were carried out in 96-well plates with fluorogenic substrates as follows: (i) DPAP1 with 200 μM Pro-Arg-amidomethylcoumarin (PR-AMC; Bachem) as described previously (10); (ii) PFA-M1 with 200 μM Ala-AMC (Sigma) in 50 mM Tris-HCl, pH 7.5 (Ala-AMC is efficiently hydrolyzed by PFA-M1 but not PFLAP (15)); (iii) PAPP with 200 μM lysine(N²-2-aminobenzoyl)-Pro-Pro-4-nitroanilide (Bachem) (28) in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM MnCl₂, 10 μM bestatin; (iv) PFPD in a coupled assay (29) containing 100 μM Asp-Ala-Pro-β-naphthylamide and 0.2 units/100 μl of rabbit kidney dipetidyl peptidase IV in 50 mM Tris-HCl, pH 7.5, 10 μM bestatin. When enzyme extracts were prepared with Triton X-100 (Fig. 3), 0.1% Triton X-100 was included in all assays. Lactate dehydrogenase (LDH) activity was detected spectrophotometrically at 650 nm in a lactate oxidation assay (30) containing 100 mM Tris-HCl, pH 9.2, 100 mM lactate, 1 mM 3-acetylpyridine adenine dinucleotide, 2 mM phenazine ethosulfate, 1 mg/ml nitro blue tetrazolium, and 0.2% Triton X-100. Enzyme rates were calculated by linear regression using Kaleidagraph 4.0.

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Aminopeptidase (PfLAP) is restricted to hydrophobic P1 residues (15). The convention of Schechter and Berger (32) is used to designate the positions of residues in the substrate and the corresponding binding sites in the peptidase. Aminopeptidase P (PfAPP) and aspartyl aminopeptidase (PfDAP) have not yet been characterized but are predicted by homology to have highly restricted P1’ and P1 specificities, respectively (Table 1). Thus, each P. falciparum AP appears to have a distinct specificity range, and together they represent the apparent sum total of AP activities that could play a role in peptide catabolism. Four methionine APs encoded in the genome (16) were not considered likely to participate directly in oligopeptide catabolism, as they function in the co-translational removal of methionine from the N termini of newly synthesized proteins (33). These methionine APs were therefore not analyzed in this study.

**Aminopeptidase Localization**—Stable parasite lines expressing each AP in Table 1 as a fusion with the yellow fluorescent protein (YFP) allele Citrine were generated. As depicted in Fig. 1A, each chromosomal AP gene was modified to encode an AP-YFP fusion by homologous recombination with a transfected episome (see also supplemental Fig. S1). Transcription of each single copy AP-YFP chimera was controlled by the respective endogenous AP promoter; therefore, physiologically relevant patterns of AP expression were expected to be maintained, as observed previously with this approach (21).

All four AP-YFP parasite lines exhibited fluorescence during the asexual erythrocytic cycle (Fig. 1B and supplemental Figs. S2–S5). Two APs were observed in the parasite’s food vacuole, the site of hemoglobin catabolism: PfAPP (Fig. 1B and supplemental Fig. S2) and PfA-M1 (Fig. 1B and supplemental Fig. S3). Interestingly, both of these APs also accumulated in a second compartment as follows: the cytosol (PfAPP) and the nucleus (PfA-M1). The two other APs (PfDAP and PfLAP) appeared in the cytosol throughout the erythrocytic cycle (Fig. 1B and supplemental Figs. S4 and S5). Both PfDAP and PfLAP were clearly excluded from the nucleus. In addition to its cytosolic location, PfLAP appeared in punctate structures in mature schizonts (Fig. 1B). The cellular location of these fluorescent spots is not clear; they did not co-localize with markers for rhoptries, micronemes, the endoplasmic reticulum, the Golgi apparatus, the apicoplast, or the mitochondrion (supplemental Fig. S6). Localization assignments for all APs are summarized in Table 1.

SDS-soluble protein extracts from each AP-YFP parasite line were subjected to immunoblotting with an anti-YFP antibody to assess the number and sizes of YFP-containing polypeptides (Fig. 2). Both PfLAP-YFP and PfDAP-YFP were present as intact fusions in their respective extracts. PfLAP-YFP exhibited a higher mobility than would be expected for the full-length fusion protein (Fig. 2). The apparent decrease in size of ~15 kDa is consistent with loss of the N-terminal asparagine-rich region (15). Interestingly, removal of this region was required for the expression of active recombinant PfLAP (15); our data suggest that the asparagine-rich region is absent from PfLAP in vivo. Intact fusion protein was also observed in extracts of PfAPP-YFP and PfA-M1-YFP parasites; however, a 25-kDa YFP species was present that presumably reflects cleavage of YFP from the fusions. This is not a surprising outcome considering that both of these AP-YFP fusions are targeted to the FV and exposed to high levels of aspartic and cysteine endopeptidase activities. Cleavage of GFP was previously observed with fusions to the resident FV peptidases PM II and dipeptidyl aminopeptidase 1 (DPAP1 (10, 21)), and removal of GFP from a PM II-GFP fusion was found to occur simultaneously with proenzyme maturation, an event that is presumed to occur in the FV (21). Similarly, cleavage of YFP from PM II was observed in a parasite line expressing PM II-YFP (Fig. 2). There is no
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FIGURE 1. RTPCR analysis of PfAPP, PfA-M1, and PfDAP. Primers specific for PfAPP, PfA-M1, and PfDAP are indicated by arrows. A, primers for PfAPP. B, primers for PfA-M1. C, primers for PfDAP. Yields are shown as a percentage of total primers added. X, untransfected parasites; Y, parasites expressing PfAPP-YFP; Z, parasites expressing PfA-M1-YFP; A, parasites expressing PfDAP-YFP.

FIGURE 2. Immunoblot analysis of extracts of parasite lines expressing YFP fusions. Proteins from $10^8$ trophozoite and schizont stage parasites from each indicated parasite line were extracted in reducing SDS-containing sample buffer, resolved on 10% polyacrylamide gels, and analyzed by immunoblotting with anti-YFP (upper panel). The position of free YFP is indicated with an arrowhead. Sizes of markers are indicated on the right. To indicate relative loading levels, the blot was stripped and reprobed with anti-BiP (lower panel). Expected sizes for the fusions in kDa are as follows: PM II-YFP, 81; PfA-M1-YFP, 152; PIAPP-YFP, 118; PFLAP-YFP, 97; PFDAP-YFP, 95. Calculated sizes for PfA-M1 and PfAPP lack the sequence up to and including the signal peptide. Note the higher-than-expected mobility of PFLAP, which migrates at ~82 kDa.

To further confirm the authenticity of the YFP fusion localizations, transgenic parasite lines were created that expressed each AP with a hemagglutinin (HA) tag (Fig. 1A). Each AP was then localized by indirect immunofluorescence in aldehyde-fixed parasites. The presence of cytosolic pools of PfAPP, PFLAP, and PFDAP was confirmed (data not shown), as was the nuclear localization of PfA-M1 (Fig. 1C). We did not observe vacuolar pools of PfAPP and PfA-M1, probably because of degradation of the HA tag by food vacuole peptidases. Consistent with this idea, a control parasite line expressing an HA-tagged version of the resident FV endopeptidase PM II also did not yield food vacuole immunofluorescence (data not shown). In parasites expressing PFLAP-HA, we did not observe the punctate structures described above for PFLAP-YFP. The anti-HA antibody did not label untransfected parasites.

Aminopeptidase Activities in the Food Vacuole—As the P. falciparum FV has been previously reported to be devoid of aminopeptidase activity (12), we sought to directly demonstrate the presence of PfAPP and PfA-M1 in this organelle. Extracts of enriched FVs and intact trophozoites were assayed for PfAPP, PfA-M1, and PFDAP activities using substrates specific for each (no substrate for specific detection of PFLAP was available). The resident FV exopeptidase PDP1 was selected as a positive control for FV enrichment. Ideally, enzyme rates would be reported as specific activities following normalization against total protein concentration. However, the very low yields of enriched food vacuoles precluded determination of protein concentration in this extract. Instead, activity of the cytosolic enzyme lactate dehydrogenase was used for normalization. Considering that LDH activity is greatly depleted from FV preparations (supplemental Table S2), the FV: trophozoite ratio of LDH-normalized peptidase activity provides a quantitative indicator of the enrichment of peptidase activity in FV extract.

The positive control DPAP1 was enriched 29-fold in FV extract (Fig. 3A and supplemental Table S2). PfA-M1 and APP activities were similarly highly enriched in FV extract (21- and 42-fold increase in peptidase:LDH activities over trophozoite extract, respectively), which is consistent with the AP-YFP localization experiments (Fig. 1B). PfA-M1 activity in FV extract was almost completely inhibited by the AP inhibitor bestatin (Fig. 3B), as expected (12). Importantly, PFDAP activity, which was localized to the cytosol, was not significantly enriched in FV extract (Fig. 3A).

Exopeptidase Synergy in the Catabolism of Proline-containing Peptides—The FV dipeptidase PDP1 does not cleave substrates with proline in the second (P2) position but does hydrolyze substrates with proline in the first (P1) position (10). Given the vacuolar location of PFAPP, we hypothesized that this exo- peptidase could work synergistically with DPAP1 in the FV to degrade oligopeptides with an N-terminal X-Pro sequence: the X-Pro aminopeptidase activity of PFAPP would transform the oligopeptide from a very poor DPAP1 substrate to a good one. To test this idea, we examined the hydrolysis of the fluorescent reporter βNA from the substrate Ala-Pro-Ala-βNA. PFAPP-mediated release of the N-terminal Ala would produce Pro-Ala-βNA, which should be readily hydrolyzed to Pro-Ala and βNA by PDP1 (Fig. 4A).

βNA was liberated from Ala-Pro-Ala-βNA during incubation with an extract of enriched FVs at pH 5.5 (Fig. 4B). The lag in the appearance of βNA is consistent with the involvement of two enzymes in βNA production. Release of βNA was blocked by the irreversible PDP1 inhibitor PR-FMK (Fig. 4B) but not by inhibitors of FV plasmepsins (pepsatin), falcipains (E-64), or...
PfA-M1 (bestatin; Fig. 4B and data not shown). Lack of inhibition by bestatin rules out the possibility (albeit unlikely) of DPAP1 cleavage of Ala-Pro-Ala-βNA to yield Ala-βNA, followed by PfA-M1-catalyzed hydrolysis of Ala-βNA to Ala and βNA.

To directly demonstrate a role for PfAPP in βNA release, DPAP1 was enriched by anion-exchange fractionation of trophozoite extract and was mixed with purified recombinant PfAPP (rPfAPP) at pH 5.5. On its own, neither enriched DPAP1 nor rPfAPP liberated βNA from Ala-Pro-Ala-βNA (Fig. 4C). When rPfAPP was mixed with enriched DPAP1, however, βNA appeared at a rate that was dependent on the amount of rPfAPP added (Fig. 4C). The appearance of βNA was blocked by PR-FMK but not E-64 or bestatin (data not shown). Together, these data suggest a role for PfAPP in “unblocking” peptides with an N-terminal X-Pro sequence, which permits further degradation by vacuolar (or cytosolic) exopeptidases.

**AP Gene Disruption**—To assess the importance of the four APs for replicative success in the erythrocyte, we attempted to independently disrupt each AP open reading frame. A single crossover strategy was adopted (34), whereby integration of an episome into each chromosomal AP coding sequence would result in the expression of a truncated protein fused to YFP (Fig. 5A). We elected to express the truncated polypeptides as YFP fusions so that their cellular locations could be assessed and compared with those of the full-length proteins. For all four APs, truncation was expected to result in an inactive protein because of the loss of one or more essential residues from the truncated sequences (supplemental Fig. S7).

Recombination between episomal and chromosomal AP sequences was not detected when PfA-M1, PfLAP, or PfAPP was targeted for disruption with three drug cycles (Fig. 5B and supplemental Fig. S8). This result suggests that the loss of any of these three APs confers a significant disadvantage during erythrocytic growth. Although it is not possible to conclude from this experiment that the targeted genes code for essential proteins, it is clear that genes encoding proteins that are not required to maintain wild-type growth rates can readily be disrupted by the single crossover approach employed here. Examples include the cysteine proteases falcipain-1 and -2 (35–37) and each of the four food vacuole plasmepsins when targeted individually (38).

In contrast, we were able to isolate clonal parasite lines carrying a disrupted PfDAP gene (Fig. 5B). The cellular location of the truncated PfDAP-YFP fusion was assessed in clone C2. As with the full-length PfDAP-YFP parasite line, YFP fluorescence in clone C2 was cytosolic (supplemental Fig. S9). We also observed areas of bright YFP fluorescence that may arise from aggregation of improperly folded truncated PfDAP-YFP (supplemental Fig. S9). These aggregates were never observed with full-length PfDAP-YFP.

To confirm the loss of active PfDAP in the disruption clone C2, activity assays were carried out with soluble extracts of C2 parasites and compared with those of wild-type 3D7 parasites and of parasites expressing full-length PfDAP-YFP. Because PfDAP does not hydrolyze simple fluorogenic aminoacyl substrates (29), PfDAP activity was measured in a coupled assay with the substrate Asp-Ala-Pro-βNA in the presence of rabbit
dipeptidyl peptidase IV. Cleavage of the Asp residue by PfDAP converts the tripeptide into a dipeptide-βNA species, and free β-naphthylamide is subsequently released by DPP IV. When lactate dehydrogenase and PfA-M1 activities were assayed and normalized to numbers of parasites, comparable levels were found in all three lines. However, with clone C2 extract, the rate of hydrolysis of β-naphthylamine from Asp-Ala-Pro-βNA was less than 10% that observed in wild-type and PfDAP-YFP-expressing parasites (Fig. 5C). This result confirms that PfDAP is an active aspartyl aminopeptidase and is consistent with the observed disruption of the PfDAP allele. The low amount of cleavage of the coupled assay substrate Asp-Ala-Pro-βNA in clone C2 extract may reflect weak activity of other plasmodial peptidases against this substrate. As an essential histidine residue is absent from truncated PfDAP (supplemental Fig. S7), we believe that no residual activity exists in the truncated PfDAP protein.

**Effect of Exogenous Amino Acids on Bestatin Toxicity**—The fungal metabolite bestatin is a potent inhibitor of APs, including PfA-M1 and PfLAP (13, 15), but not PfAPP or PfDAP.4 Bestatin has been shown to have anti-malarial activity against *in vitro* cultures (11, 39); however, bestatin toxicity is typically assayed in the presence of exogenous pools of all 20 essential amino acids. These exogenous amino acids may compensate for the inhibition of PfA-M1 and PfLAP in parasites by allowing them to overcome a block in endogenous amino acid production.

Recently, it has been demonstrated that hemoglobin catabolism can supply the amino acids necessary for parasite growth with the exception of isoleucine, which is absent in the human hemoglobin sequence (22). Parasite biosynthetic pathways can also provide Glu, Asp, and Ala (40). We asked whether parasites that are forced to rely on hemoglobin catabolism and endogenous biosynthesis to provide amino acids for growth are more susceptible to bestatin than those that are cultured in the presence of exogenous amino acids. Concentration-response curves for inhibition of parasite replication by bestatin in RPMI medium containing isoleucine as the sole amino acid (I medium) or in RPMI containing all 20 amino acids (All AA medium) are depicted in Fig. 6A. A small enhancement of bestatin potency was observed in I medium over All AA medium, with EC₅₀ values of 0.9 and 1.8 μM, respectively.

The development of ring-stage parasites in either I or All AA medium that were treated with bestatin at a concentration 14-fold over the respective EC₅₀ was compared with that of parasites that were deprived of all amino acids in the medium.

4 K. Bompiani, S. Geherin, and M. Klemba, unpublished observations.

**FIGURE 5. Targeted disruption of AP genes.** A, schematic diagram of the single crossover strategy for the disruption of AP genes. Upon integration of the episome, truncated APs would be expressed from the endogenous AP promoter as YFP fusions. B, Southern blot analysis of AP gene disruption experiments. Digested genomic DNA from transfected parasites that were cycled off and on drug two (PfDAP clone C2) or three times (PfLAP, PfAPP, and PfA-M1) and from untransfected parental 3D7 parasites was probed with AP-specific probes. The positions of the wild-type (wt), episomal (epi), and post-recombination (int1 and int2) fragments are indicated with arrowheads. Expected fragment sizes are depicted in supplemental Fig. S8. In the PfDAP panel, the wild-type fragment is absent from clone C2, and two new bands have appeared that correspond to those from the disrupted PfDAP locus. In the other panels, the wild-type fragment is intact following drug cycling.

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Development was assessed by quantitation of the DNA content of parasite populations by flow cytometry, which is a sensitive indicator of progression through the blood cycle (41). Parasite populations treated with bestatin had similar DNA contents to one starved of amino acids (Fig. 6B). When the mean fluorescence intensity of each parasite population was calculated, no significant difference was observed between bestatin-treated and amino acid-starved populations (Fig. 6C). At bestatin concentrations closer to the EC50 value (i.e. 3.6 times the EC50), some parasite development was observed, as indicated by the increase in the mean YOYO-1 fluorescence intensity (Fig. 6, B and C). These data strongly suggest that peptide catabolism by APs is critical in early stages of parasite development, even when exogenous amino acids are present.

**DISCUSSION**

The prevailing model for the generation of amino acids from hemoglobin holds that peptides are exported out of the FV for hydrolysis by cytosolic aminopeptidases. In contradiction to this model, we have demonstrated that two aminopeptidases (PfA-M1 and PfAPP) reside in the FV. The presence of both enzymes in FVs isolated from parasites with wild-type AP alleles corroborates the YFP-based localization data and indicates that the FV pools of enzyme do not arise from mistargeting induced by the C-terminal YFP tag. Furthermore, the presence of a putative signal peptide near the N termini of PfA-M1 and PfAPP (Table 1) is consistent with established routes of trafficking of FV proteins through the secretory system (10, 21). We propose a revised model for hemoglobin catabolism whereby amino acids are generated from globin-derived oligopeptides in the FV lumen through the activities of PfA-M1 and PfAPP (Fig. 7). Although peptide diffusion or transport across the FV membrane may contribute to efficient hemoglobin catabolism, we assert that it is not required for the production of amino acids. Six amino acid transporters have been identified in the *P. falciparum* genome sequence (42), and one or more of these may facilitate passage of amino acids across the FV membrane.

PFA-M1 has been reported to hydrolyze a diverse set of amino acid-AMC substrates at pH 7.4, including Lys, Arg, Leu, Phe, Tyr, Ser, Asn, and Ala (13). Importantly, PFA-M1 was found to retain 40% of maximal activity at pH 5.8 (13), which is consistent with an enzymatic role in the acidic lumen (43–45) of the FV. We suggest that the role of PFA-M1 in the FV is to catalyze the release of amino acids from the highly sequence-diverse peptides produced from α- and β-globin (Fig. 7). The *Plasmodium chabaudi* ortholog of PFA-M1 is probably the AP activity detected by Slomianny et al. (46) in digestive vesicles of this parasite using an ultrastructural approach. Perhaps the most surprising and puzzling result of this study was the localization of PFA-M1 to the nucleus. This initial assignment with the increase in the mean YOYO-1 fluorescence intensity (Fig. 6, B and C). These data strongly suggest that peptide catabolism by APs is critical in early stages of parasite development, even when exogenous amino acids are present.

**DISCUSSION**

The prevailing model for the generation of amino acids from hemoglobin holds that peptides are exported out of the FV for hydrolysis by cytosolic aminopeptidases. In contradiction to this model, we have demonstrated that two aminopeptidases (PFA-M1 and PfAPP) reside in the FV. The presence of both enzymes in FVs isolated from parasites with wild-type AP alleles corroborates the YFP-based localization data and indicates that the FV pools of enzyme do not arise from mistargeting induced by the C-terminal YFP tag. Furthermore, the presence of a putative signal peptide near the N termini of PFA-M1 and PfAPP (Table 1) is consistent with established routes of trafficking of FV proteins through the secretory system (10, 21). We propose a revised model for hemoglobin catabolism whereby amino acids are generated from globin-derived oligopeptides in the FV lumen through the activities of PFA-M1 and PfAPP (Fig. 7). Although peptide diffusion or transport across the FV membrane may contribute to efficient hemoglobin catabolism, we assert that it is not required for the production of amino acids. Six amino acid transporters have been identified in the *P. falciparum* genome sequence (42), and one or more of these may facilitate passage of amino acids across the FV membrane.
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fluorescence to the parasite cytosol and the cytolic face of the FV (13, 31). The disagreement between our results in live parasites expressing PfA-M1-YFP and the data from fixed parasites is probably rooted in the difficulty in preserving the integrity of the food vacuole when parasites are fixed for immunofluorescence (47).

In contrast to PfA-M1, P. falciparum aminopeptidase P (PfAPP) possesses a highly restricted substrate specificity, cleaving N-terminal residues from peptides containing a P1’-proline (X-Pro specificity, where X is any amino acid). Because of the unusual side chain structure of proline, many exopeptidases do not readily accommodate a P1’-proline residue in their active sites; thus, a critical role for APP in the catabolism of proline-containing peptides has been proposed (48). Human α- and β-globin each contain seven proline residues; endopeptidase degradation of these polypeptides in the FV is therefore expected to yield oligopeptides with an N-terminal X-Pro sequence. We have shown that PfAPP can facilitate the further degradation of X-Pro peptides at acidic pH (for example, by DPAP1) by removing the first amino acid. This is the first report, to our knowledge, of an APP homolog residing in an acidic degradative organelle. PfAPP was also found in the cytosol throughout the erythrocytic cycle, where it likely has a similar role in the catabolism of X-Pro peptides generated by the proteasomal degradation pathway (Fig. 7). Such a housekeeping function has been proposed for the cytosolic mammalian homolog APP1 (49).

The other two P. falciparum aminopeptidases studied here, leucyl aminopeptidase (PfLAP) and aspartyl aminopeptidase (PfDAP), are located in the parasite cytosol. Our results for PfLAP are in agreement with those obtained from episcopal expression of GFP-tagged PfLAP or in immunofluorescence experiments with anti-PfLAP antibodies (15). In addition, we observed fluorescent spots in late-stage schizonts expressing PfLAP-YFP, which have not been previously reported. There appeared to be one spot per daughter nucleus, suggesting an organellar association. However, we were unable to demonstrate co-localization of these spots with markers for rhoptries, micronemes, the endoplasmic reticulum, the Golgi apparatus, the apicoplast, or the mitochondrion. Furthermore, these structures were not observed by immunofluorescence in parasites expressing HA-tagged PfLAP. Until the biological relevance of these structures is confirmed, we view them as a possible artifact stemming from the presence of the YFP tag, and we consider the role of PfLAP to be the release of amino acids from cytosolic peptides (Fig. 7), in agreement with previous reports (14, 15).

Through targeted gene disruption experiments, we have identified three APs that appear to be required for efficient parasite replication. These results validate the view that APs perform critical functions during erythrocytic growth (11), and identify a cohort of APs that are promising targets for antimalarial drug development. Given the proposed role for aspartyl AP homologs in the degradation of peptides containing P1 Asp or Glu residues, and their apparent widespread distribution in eukaryotes from yeast to humans (29), it was surprising to find that PfDAP was dispensable for blood-stage replication.

The PfDAP gene truncation clone grows in I medium, which indicates that this AP is not required to produce Asp and Glu for parasite protein synthesis. This observation is consistent with the known capacity of malaria parasites to synthesize these two amino acids from metabolic intermediates (40). We are investigating whether there is a fitness cost associated with the loss of PfDAP. Alternatively, this enzyme may function in other parts of the parasite life cycle.

The AP inhibitor bestatin and its derivatives nitrobestatin and bestatin methyl ester are significantly less potent in inhibiting parasite growth in culture than they are against their presumed targets in vitro (11, 14, 15). As both PfA-M1 and PfLAP are thought to be intracellular targets of bestatin (13, 15), we predict that this inhibitor will block the release of amino acids from both the FV and proteasomal protein degradation pathways. Parasites are known to utilize exogenously supplied amino acids for protein synthesis (50); we therefore examined whether the presence of all 20 essential amino acids in standard culture medium was compensating for the loss of AP activity in bestatin-treated parasites. In I medium, all amino acids except isoleucine must be derived from biosynthetic pathways (which can provide Asp, Glu, and Ala (40)) or from parasite peptide catabolism, of which hemoglobin catabolism is a major component. A 2-fold decrease in bestatin EC50 was seen in I medium compared with that in medium containing all 20 essential amino acids. Clearly, the presence of exogenous amino acids does not have a large mitigating effect on bestatin toxicity. A similar 2-fold drop in EC50 has been observed with aspartic and cysteine protease inhibitors in I medium (22). This consistent downward shift in EC50 in I medium may indicate a greater sensitivity to changes in flux through amino acid-producing pathways; alternatively, it may reflect a reduced ability of the parasite to tolerate stress.

When the development of bestatin-treated parasite populations was compared with an untreated one starved of all amino acids (and unable to synthesize protein because of the absence of isoleucine), no significant differences in the mean levels of DNA were observed. These data imply that the catabolism of peptides to amino acids is required for development beyond the ring/young trophozoite stage, even in the presence of exogenous amino acids. This result is consistent with the observation that hemoglobin catabolism begins early in the erythrocytic cycle (51). Although the requirement for AP activity in I medium is easy to rationalize based on the need for amino acids for protein synthesis, an intriguing question is why the presence of exogenous amino acids does not complement the loss of AP activity. One possible explanation is that the parasite depends on APs for one or more amino acids that are not acquired from the medium in sufficient quantities. Alternatively, the accumulation of undegraded peptides may be cytotoxic. Another intriguing possibility is that the depletion of cytosolic amino acid pools impairs the transport of isoleucine (which is not present in hemoglobin) across the parasite membrane (7). Whatever the mechanism of toxicity, these observations suggest that the presence of amino acids in vivo (i.e. in host plasma)
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