Falcipain Cysteine Proteases Require Bipartite Motifs for Trafficking to the *Plasmodium falciparum* Food Vacuole*

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The *Plasmodium falciparum* cysteine proteases falcipain-2 and falcipain-3 hydrolyze hemoglobin in an acidic food vacuole to provide amino acids for erythrocytic malaria parasites. Trafficking to the food vacuole has not been well characterized. To study trafficking of falcipains, which include large membrane-spanning prodomains, we utilized chimeras with portions of the proenzymes fused to green fluorescent protein. The prodomains of falcipain-2 and falcipain-3 were sufficient to target green fluorescent protein to the food vacuole. Using serial truncations, deletions, and point mutations, we showed that both a 20-amino acid stretch of the lumenal portion and a 10-amino acid stretch of the cytoplasmic portion of the falcipain-2 prodomain were required for efficient food vacuolar trafficking. Mutants with altered trafficking were arrested at the plasma membrane, implicating trafficking via this structure. Our results indicate that falcipains utilize a previously undescribed bipartite motif-dependent mechanism for targeting to a hydrolytic organelle, suggesting inhibition of this unique mechanism as a new means of antimalarial chemotherapy.

Malaria causes hundreds of millions of illnesses annually, and *Plasmodium falciparum*, the most virulent human malaria pathogen, causes a million or more deaths each year (1). Intraerythrocytic *P. falciparum* parasites develop over 48 h from rings to more metabolically active trophozoites and then to multinucleated schizonts, which rupture host erythrocytes, releasing invasive merozoites that reinstitute erythrocytic infection. Trophozoites take up and hydrolyze hemoglobin to obtain amino acids for protein synthesis (2, 3). Hemoglobin is acquired from the erythrocyte via large endocytic vesicles called cytostomes and is degraded in the food vacuole, a lysosome-like acidic organelle (4). Hydrolysis of hemoglobin is essential for parasite survival (3).

Hemoglobin hydrolysis requires the action of multiple proteases, including falcipain cysteine proteases, plasmepsin aspartic proteases, and others (5, 6). Inhibitors of cysteine and aspartic proteases block hemoglobin hydrolysis and parasite development, suggesting that this pathway is a promising target for antimalarial chemotherapy (7). Among the four *P. falciparum* falcipain cysteine proteases (8–11), falcipain-2 and falcipain-3 appear to be the principal food vacuolar hemoglobinases (12). Disruption of the *falcipain*-2 gene led to accumulation of undegraded hemoglobin in the food vacuole, confirming that this enzyme participates in hemoglobin hydrolysis (13). Disruption of *falcipain*-3 could not be achieved, but the gene was readily replaced with a tagged functional copy, indicating that falcipain-3 is essential for erythrocytic parasites (14). The roles of falcipain-2’ (a near-identical copy of falcipain-2) and falcipain-1 are unknown; knock out of these two proteases led to no apparent alterations in erythrocytic parasites (14, 15).

The mechanism of trafficking to the *P. falciparum* food vacuole is poorly understood. In higher eukaryotes, lysosomal targeting generally requires glycosylation and trafficking is mediated by mannose-6-phosphate receptors (16). However, *P. falciparum* does not have a robust glycosylation machinery (17), and there is no evidence for trafficking mediated by this process. Zymogenic enzymes in some eukaryotes, including yeast (18) and plants (19), carry vacuolar localization signals in their prodomains. In yeast, proforms of soluble vacuolar enzymes are targeted via a receptor-mediated transport mechanism (20). In *P. falciparum*, zymogenic forms of food vacuolar falcipains and plasmepsins are membrane-bound (6, 12); it is unclear whether classical receptor-mediated transport is possible for membrane-bound proteins. Membrane-bound vacuolar proteins are trafficked by adaptor-mediated mechanisms in yeast and other eukaryotes (21). However, the classical dileucine or hydrophobic cytoplasmic motifs required for adaptor binding by many vacuolar/lysosomal proteins (21, 22) are not seen in falcipains or plasmepsins. These observations suggest that previously described mechanisms may not mediate food vacuole trafficking of *P. falciparum* proteases. Indeed, immunoelectron microscopy studies suggested that plasmepsin II initially traffics to the plasma membrane, followed by transport with erythrocyte contents in cytostomes to the food vacuole (23). However, targeting signals for plasmepsins or other food vacuole proteins have not been characterized in detail.

As a first step toward characterizing food vacuolar trafficking pathways, we sought to identify motifs that target falcipains to this organelle. We therefore created multiple chimeras linking portions of the falcipain prodomains with green fluorescent
protein (GFP). Our data reveal a novel mechanism of trafficking for the *P. falciparum* cysteine proteases, with portions of both the cytoplasmic and luminal domains of these membrane-bound proenzymes necessary for food vacuolar trafficking and alterations in targeting signals leading to trapping at the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Vent DNA polymerase (New England Biolabs) was used for all PCR reactions. Descriptions of all primers are in supplemental Table S1. The plasmid backbone used for all constructs was pSSPF2, kindly provided by Iain Wilson (24), which contains a Cam5/hDHFR/Hrp2 selection cassette and Hsp86/Hsp60-GFP/PbhD3 cassette for expression of GFP fusion proteins. pSSPF2 bearing Hsp60-GFP was digested with BglII, its ends were filled in with Klenow, and it was then digested with AvrII to release the Hsp60 fragment. The full-length prodomain of *falcipain*-2 was amplified using forward primers with BstB1/PstI/Sall and reverse primers with KpnI/AvrII sites. AvrII-digested PCR product was ligated to the digested plasmid to generate the proFP2-GFP construct under the *HSP86* promoter. A linker sequence (from the KpnI/AvrII sites) encoding for amino acids Gly-Thr-Pro-Arg is present in between the end of the prodomain coding sequence and the start of the GFP sequence in all the proFP2 constructs. For generating *falcipain*-3 constructs, the prodomain of *falcipain*-3 was amplified using forward primers with Sall and reverse primers with KpnI sites. Sall/KpnI-digested PCR product was placed in the proFP2-GFP plasmid to replace proFP2. All proFP3 constructs described in this report are under the *HSP86* promoter. proFP2-GFP, proFP2-CT-GFP, and proFP2-lum-GFP constructs were generated under both the *HSP86* and the endogenous FP2 promoter; all other proFP2 truncation/deletion constructs were generated under the endogenous promoter. To replace the *HSP86* promoter with the native *falcipain*-2 promoter, a 1.2-kb DNA sequence upstream from the start codon of *falcipain*-2 was amplified. The plasmid and product were digested with Sall and then cloned into the vector to replace the *HSP86* promoter.

For all truncation constructs the forward primer contained Sall and the reverse primer was truncation-specific, carrying a KpnI site. The PCR product and plasmid were digested with Sall and KpnI and ligated together. For specific deletions or point mutations, two sets of PCR reactions were performed to amplify the regions upstream and downstream of the deletion independently, with slight overlap at the ends. Primers were designed such that the 3′-end of the reverse primer of the upstream PCR was complementary to the 5′-end of the forward primer of the downstream PCR. The PCR products were gel-purified and mixed in equal volume. These products recombined *in vitro* and were used as a template for a secondary PCR reaction. The PCR product and plasmid were digested with Sall and KpnI and then ligated as mentioned above. Expected sequences of all of the constructs were confirmed by DNA sequencing.

**Parasite Culture and Plasmid Transfection**—All the experiments were performed in *P. falciparum* strain 3D7 (MRA151 from the Malaria Research and Reference Reagent Resource Center). Parasites were cultured in human erythrocytes at 2% hematocrit in RPMI 1640 medium (UCSF Cell Culture Facility) supplemented with 10% human serum. Synchronization was maintained by serial treatment with 5% d-sorbitol. Early ring-stage parasites (100–µl packed cell volume, 4–6% parasitemia) were transfected with 80–100 μg of plasmids (prepared with a Qiagen maxiprep kit) in a 0.2-cm cuvette by using a Gene Pulser II (Bio-Rad) at 0.31 kV, 950 μF, and maximum resistance (13, 25). Transfectants were selected with WR99210 (2.5 mM; Jaccobus Pharmaceuticals), which was added 48 h after the electroporation and maintained thereafter. One week after emergence of parasites (3–4 weeks after transfection), WR99210 was increased to 5.0 mM, and transfectants were maintained at this concentration of the drug henceforth.

**Microscopy**—Microscopy was performed on live trophozoites at 22–28 h after erythrocyte invasion unless indicated otherwise. Parasites were washed twice in serum-free RPMI 1640 medium and once in PBS and then allowed to adhere to poly-L-lysine-coated coverslips for 30 min in the presence of Hoechst 33342 (10 μM; Molecular Probes) to visualize the nucleus. Unattached cells were gently washed off using PBS. A coverslip was placed on the barely wet slide, and the cells were imaged immediately using a Zeiss Axioplan epifluorescence microscope using a 63× objective lens. A Hamamatsu CCD camera was used to capture images, and the images were processed using Openlab software (Improvision) for artificial coloring, contrast adjustment, and overlapping images. For Fig. 1, confocal microscopy was performed as mentioned above using a Zeiss LSM 510 META confocal microscope under a 100× objective.

For immunofluorescence microscopy, trophozoites washed with 1× PBS (as mentioned above) were allowed to adhere to poly-L-lysine-coated slides (Sigma) for 30 min. Excess unattached cells were washed off with 1× PBS. The cells were fixed for 30 min with 3% formaldehyde + 0.5% glutaraldehyde in PBS at room temperature, followed by 15 min of permeabilization with 0.1% Triton X-100 in PBS. After three washes with PBS, the cells were incubated with 10 mM NH4Cl for 10 min, followed by blocking for 3 h with 3% bovine serum albumin in PBS. 1/150 dilution rat anti-FP2 or FP3 (12) or 1/50 rabbit anti-PfCRT (26) in blocking solution were added to the slides and incubated for 2 h. After several washes, 1/300 dilution Cy3-conjugated anti-rat antibody (Jackson ImmunoResearch Laboratory) or 1/300 Alexa-594-conjugated anti-rabbit antibody (Molecular Probes) was added to the slide for 45 min along with 10 μM Hoechst 33342. Cells were washed thoroughly and mounted using ProLong gold anti-fade reagent (Molecular Probes) and visualized under a 100× objective in a Nikon Optiphot epifluorescence microscope.

Immunoelectron microscopy was performed as described elsewhere (12). In brief, transfected trophozoites were purified using a 63% Percoll gradient after washing twice with serum-free RPMI medium. Concentrated parasites were fixed for 2 h at room temperature with 3% formaldehyde (Electron Microscopy grade) and 0.01% glutaraldehyde. Fixed para-

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3 The abbreviations used are: FP, falcipain; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HSP, heat shock protein.
sites were washed twice with PBS, and electron microscopy was performed at the UCSF Cell Imaging core facility using standard protocols (12). Rabbit anti-GFP antibody (Molecular Probes) was used at a 1/1000 dilution and rat anti-falcipain-2 (12) at a 1/500 dilution for immunodetection.

RESULTS
The Prodomains of Falcipain-2 and Falcipain-3 Are Sufficient to Direct Trafficking to the Food Vacuole—To test whether the prodomains of falcipain-2 and falcipain-3 are sufficient for food vacuolar targeting, we generated constructs encoding GFP fused to the C-terminal ends of the falcipain-2 and falcipain-3 prodomains (proFP2-GFP, proFP3-GFP), with expression driven by the HSP86 promoter, and plasmids bearing these constructs were transfected into P. falciparum (Fig. 1a). Expression of GFP fusions of expected sizes was confirmed by Western blots (supplemental Fig. S1). GFP fusion proteins were targeted to the food vacuoles of young trophozoites, as evidenced by co-localization with hemoglobin, a breakdown product of hemoglobin in this compartment, when visualized using confocal microscopy (Fig. 1b and c). Immunoelectron microscopy confirmed co-localization of proFP2-GFP (15-nm gold particles) and native falcipain-2 (10-nm gold particles) in the food vacuole (Fig. 1d). Co-localization of proFP2-GFP and proFP3-GFP was also observed with antibodies directed against the endogenous food vacuolar proteins falcipain-2, falcipain-3 (12), and the P. falciparum Chloroquine Resistance Transporter (PfCRT) (26) (Fig. 2). With some young trophozoites, we also observed proFP2-GFP in discrete structures both on the parasite membrane and in the cytoplasm (Fig. 1e and supplemental Fig. S2). Addition of Brefeldin A, a compound that blocks traffic from the endoplasmic reticulum to the Golgi compartment, led to perinuclear localization of proFP2-GFP, suggesting accumulation in the membrane of the endoplasmic reticulum (Fig. 1g), as expected for a protein normally entering the secretory pathway.

It is known that during the 48-h intraerythrocytic life cycle of the malaria parasite, timing of expression is critical for proper protein targeting and function (27). To mimic native gene expression, we also generated a proFP2-GFP construct in which expression was driven by the endogenous falcipain-2 promoter. No differences were observed in the localization pattern between cells with expression driven by the HSP86 or falcipain-2 promoter.

FIGURE 1. Localization of proFP-GFP wild type constructs. a, structure of the proFP2-GFP construct. Cytoplasmic (cytosol), transmembrane (TM), and lumenal portions of the prodomain are labeled. Confocal microscopic images show expression of proFP2-GFP (b) under the falcipain-2 promoter and proFP3-GFP (c) under the HSP86 promoter in young trophozoites. For this and other figures the panels show, from left to right, GFP, transmission light microscopy (black hemozoin pigment identifies the food vacuole), Hoechst staining of DNA, and a merged image. d, double immunogold labeling of an intraerythrocytic trophozoite transfected with a plasmid encoding proFP2-GFP. Native falcipain-2, labeled with antibody against the mature protease, is identified with 10-nm gold particles (red arrowheads) and GFP, labeled with anti-GFP antibody, is identified with 15-nm gold particles (black arrows). The bar indicates 0.6 μm. e, single immunogold labeling of an intraerythrocytic trophozoite transfected with proFP2-GFP and labeled with anti-GFP antibody. The arrow indicates a probable cytostome, shown in close-up in panel f. The bars indicate 0.5 (e) and 0.25 (f) μm. g, proFP2-GFP (under the falcipain-2 promoter) after 5 h of incubation with Brefeldin A, beginning at 22 h post-invasion and visualized using an epifluorescence microscope.
pain-2 promoters (supplemental Fig. S2), and fluorescence intensity was stronger with the endogenous promoter. Subsequent studies of falcipain-2 localization therefore utilized constructs with the endogenous promoter.

The Cytoplasmic Portions of the Falcipain-2 and Falcipain-3 Prodomains Are Not Sufficient for Food Vacuolar Trafficking—Falcipain-2 and falcipain-3 are type II membrane proteins with prodomains containing N-terminal cytoplasmic portions and C-terminal luminal portions that flank a membrane-spanning domain (Fig. 1a). To determine whether individual domains could efficiently target GFP to the food vacuole, we generated additional constructs. Trophozoites expressing only the cytoplasmic and transmembrane portions of the prodomain fused to GFP (proFP2-CT-GFP and proFP3-CT-GFP) displayed predominant plasma membrane GFP fluorescence, seen as bright fluorescent rims on the periphery of intraerythrocytic parasites (Fig. 3a). We also observed some perinuclear GFP in these parasites. Thus, the cytoplasmic portion of falcipain-2 or falcipain-3 is not sufficient for food vacuolar trafficking but targets to the plasma membrane. Trophozoites expressing only the luminal portion of the prodomain fused to GFP (proFP2-lum-GFP and proFP3-lum-GFP) displayed fluorescence throughout the cytoplasm (Fig. 3b), as expected for proteins lacking a signal sequence directing entry into the secretory pathway. Our results here suggest that trafficking pathways for falcipain-2 and falcipain-3 are similar and that trafficking includes vesicular transport via the plasma membrane.

Falcipain-2 Trafficking Is Driven by Both Lumenal and Cytoplasmic Targeting Motifs—We next focused our efforts on characterizing trafficking signals in the falcipain-2 prodomain, as the robust transport of proFP2-GFP to the food vacuole allowed us to easily distinguish phenotypes. To this end, we generated serial truncations of the luminal domain, yielding constructs encoding partial prodomains with between 65 and 225 luminal amino acids, in each case fused to GFP at the C terminus (Fig. 4). GFP fusion proteins of expected size were produced for each construct (supplemental Fig. S1). Trophozoites expressing proFP2–65-GFP, proFP2–75-GFP, and proFP2–84-GFP displayed a fluorescence pattern identical to that of proFP2-CT-GFP, with distinct plasma membrane localization (supplemental Fig. S3, a–c). Trophozoites expressing proFP2–95-GFP showed an intermediate phenotype, with a majority of cells (60–80%) showing exclusive plasma membrane fluorescence (Figs. 5a and 6a), and the rest displaying fluorescence in both the plasma membrane and the food vacuole. Most trophozoites (>70%) expressing proFP2–105-GFP showed exclusive food vacuolar fluorescence (Figs. 5b and 6a), and the rest displaying fluorescence in both the plasma membrane and the food vacuole. Most trophozoites (>70%) expressing proFP2–105-GFP showed exclusive food vacuolar fluorescence (Figs. 5b and 6a), and the remainder of cells showed both food vacuole and plasma membrane fluorescence (Fig. 5c). Larger constructs (proFP2–115-GFP, proFP2–120-GFP, proFP2–173-GFP, and proFP2–225-GFP) were efficiently transported to the trophozoite food vacuole (supplemental Fig. S3, d–g). Localization was not uniform among all parasites for each construct, but quantification of GFP localization patterns in trophozoites demonstrated predominant food vacuole localization for proFP2-GFP and all larger constructs (Fig. 6a).

Our results suggest that the luminal trafficking motif for falcipain-2 lies between amino acids 84 and 105. Most amino acid...
acids in this region are identical between falcipain-2, falcipain-2', falcipain-3, and homologues from several other plasmodial species (Fig. 7b). To further evaluate the roles of these residues in trafficking, we generated constructs with deletion of amino acids 81–110 (proFP2-Δ81–110-GFP) or 95–110 (proFP2-Δ95–110-GFP) fused to GFP (Fig. 4). Trophozoites expressing proFP2-Δ81–110-GFP predominantly displayed patches of fluorescence on the parasite plasma membrane, a phenotype that had not previously been seen (Fig. 5d). Trophozoites transfected with proFP2-Δ95–110-GFP displayed a similar pattern of fluorescence on the plasma membrane (supplemental Fig. S4b), but more commonly localization of GFP to the parasite food vacuole (supplemental Fig. S4c). Additionally, we mutated conserved lysines in the region of interest to glutamic acid. Trophozoites expressing K95E-GFP resembled those expressing proFP2-Δ95–110-GFP, with some patches of fluorescence on the plasma membrane, but principally localization to the food vacuole (supplemental Fig. S4c). In contrast, trophozoites expressing the triply mutated construct proFP2-K89,90,95E-GFP predominantly had patches of fluorescence on the plasma membrane with or without food vacuolar fluorescence (Fig. 5e), whereas ~35% of trophozoites showed exclusive food vacuolar GFP localization. Taken together, our results demonstrated a critical role in trafficking for residues 84–105 within the lumenal falcipain-2 prodomain, suggesting that positively charged residues are necessary for proper trafficking, and offered further support for the conclusion that falcipains are transported to the food vacuole via the plasma membrane.

We wished also to explore roles of amino acids in the pro-falcipain-2 cytoplasmic domain in food vacuole trafficking, as motifs on the cytoplasmic side of membrane-bound proteins mediate vacuolar targeting in other organisms (21, 22). We therefore generated constructs with deletions in the falcipain-2 cytoplasmic domain (Fig. 4). Trophozoites expressing proFP2-Δ2–15-GFP showed food vacuolar fluorescence (supplemental Fig. S4d). Those transfected with proFP2-Δ5–25-GFP displayed patches of plasma membrane fluorescence in nearly all cells (Fig. 5f), and those transfected with proFP2-Δ16–30-GFP showed patches of plasma membrane fluorescence (supplemental Fig. 5e) and/or food vacuole fluorescence (supplemental Fig. S4f). These results indicate that, in addition to the luminal domain characterized above, cytoplasmic residues 16–25 are also required for trafficking to the food vacuole.

All results described above are for trophozoite-stage parasites. Initial results suggested differential trafficking of some constructs depending on the life cycle stage that was studied. To more systematically address this area, for select constructs we evaluated localization of fusion proteins over the course of the erythrocytic life cycle. Interestingly, for both proFP2–75-GFP and proFP2–95-GFP, young trophozoites showed primarily plasma membrane localization as noted above, but food vacuole localization increased with maturation, and over 75% of late schizonts had only food vacuolar localization of GFP (Fig. 6,

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**FIGURE 3. Localization of partial prodomain constructs.** Trophozoites transfected with the indicated constructs a, CT-GFP, b, lum-GFP (HSP86 promoters), as diagrammed in the schematics, were observed by epifluorescence microscopy. Panels are as described for Fig. 1. Scale bar, 5 μm.

**FIGURE 4. Localization phenotypes observed with falcipain-2 constructs.** All constructs are diagrammed, and predominant phenotypes in early trophozoites are indicated. Diagonal lines indicate positions of internal deletions. Asterisks indicate locations of introduced point mutations (all Lys to Glu).
b and c). With qualitative assessments of mature schizonts, some localization of GFP to the food vacuole was seen with all constructs in late schizonts, including constructs that localized almost exclusively to the plasma membrane in younger parasites (Fig. 6d). These data suggest a default pathway for targeting proteins to the food vacuole in mature parasites. However, because native falcipain-2 is expressed predominantly in trophozoites (12) and because the native promoter led to expression of labeled constructs consistent with that of the native protease, our results showing varied trafficking of constructs in trophozoites are most informative regarding the biologically relevant food vacuole-trafficking pathway.

DISCUSSION

We have investigated the molecular determinants of trafficking of falcipain cysteine proteases to the food vacuole of P. falciparum. We show that falcipain-2 and falcipain-3 contain the information required for food vacuolar trafficking in their prodomains, as is the case for many other eukaryotic proteins that are trafficked to vacuoles. However, surprisingly, this trafficking signal is bipartite in nature, with motifs in both the luminal and cytoplasmic portions of the membrane-bound prodomains. In the luminal portion, amino acids 84–105 are needed for food vacuolar targeting, with lysine residues within this domain contributing to efficient trafficking. In the cytoplasmic portion, amino acids 16–25 are also necessary for proper trafficking. This is, to our knowledge, the first demonstration of a requirement for signals on both sides of a transmembrane domain for trafficking of a vacuolar protein. Most constructs that did not traffic to the food vacuole were localized to the parasite plasma membrane, with at least two patterns seen, showing diffuse or discontinuous membrane localization. These results suggest that the bipartite falcipain-trafficking signal directs an ordered set of events leading to localization at discrete regions of the plasma membrane followed by trafficking to the food vacuole.

Although similar, results for trafficking of falcipain-2 and falcipain-3 were not identical. Prior studies have shown that, although these vacuolar hemoglobinases are biochemically very similar, they differ in some important respects. First, falcipain-2 is expressed earlier in the life cycle than falcipain-3 (12). Second, knock out of the falcipain-2 gene led to a transient block in hemoglobin hydrolysis, but parasites remained viable (13); parasites could not tolerate knock out of falcipain-3 (14). Third, autoprocessing of falcipain-2 occurs over a wide pH range, but falcipain-3 requires acidic pH (9, 11). Fourth, the proform and processing intermediates of falcipain-3 are much longer lived than those of falcipain-2 (12). Adding to these data, our new studies show that falcipain-3 is transported to the food vacuole with slower kinetics than is falcipain-2, such that falcipain-3 localizes much more commonly to subcellular structures (also seen with labeling of native falcipain-3) (12) that likely represent vesicles containing trafficking intermediates.

Using point mutations we demonstrated that lysines in the luminal targeting motif contribute toward proper and efficient targeting to the food vacuole. These positively charged amino acids might be crucial for the structural stability of the prodomain and/or its interactions with proteins required for proper trafficking. Similarly, positively charged residues play a key role in trafficking the cysteine protease cruzain to the lysosome/endosome compartment in the protozoan Trypanosoma cruzi (28). On the cytoplasmic side of the falcipain-2 prodomain, a Phe-Val motif between residues 15 and 25 is conserved across homologues of falcipains (Fig. 7a). A hydrophobic trafficking motif, YXXΦ, on the cytoplasmic side of membrane proteins has been shown to bind to adaptor molecules, which traffic proteins to lysosomal and endosomal compartments (21), but this motif is not found in falcipains. Alternatively, the Phe-Val motif of falcipains might interact with receptors or adaptors to be targeted to the food vacuole. It is not clear whether similar mechanisms of targeting apply to other P. falciparum food vacuole proteins. In the case of aspartic proteases, plasmepsin II appears to follow a similar pathway, with evidence for transit through the cytostome (23), but similarities of prodomain sequences at sites shown to be important for falcipain trafficking are not obvious. A recent report showed that the first 70 amino acids of plasmepsin IV (including 39 cytoplasmic, 20 transmembrane, and 11 luminal residues) are sufficient for food vacuolar targeting (29). It is possible that both cytoplasmic and luminal motifs are also required for targeting of plasmepsin.
IV, but additional studies will be needed for full characterization of plasmpedin-trafficking motifs.

Our results suggest a model for the trafficking of falcipain-2 and falcipain-3. As expected, the transmembrane domains of falcipain-2 and falcipain-3 are required for entry into the endoplasmic reticulum and secretory pathway; absence of transmembrane domains led to localization of GFP chimeras to the cytoplasm. Once in the endoplasmic reticulum, the proteins appear to traffic to the plasma membrane. At the plasma membrane falcipain-2 and falcipain-3 appear to be endocytosed in a process requiring the presence of both cytoplasmic and luminal prodomain-trafficking motifs, followed by vesicular transport to the food vacuole. Trafficking to the food vacuole via the plasma membrane was previously proposed for plasmepsin aspartic proteases (23). Our demonstration of arrest of trafficking mutants at the plasma membrane offers strong evidence that falcipain-2 and falcipain-3 similarly traverse the plasma membrane en route to the food vacuole.

Evidence for our model is indirect, and it remains possible that falcipains are trafficked in a fashion similar to that of vacuolar proteins in yeast, with targeting from the endoplasmic reticulum to Golgi to food vacuole (20), and that a defect in sorting at the Golgi led to plasma membrane localization of some of our constructs. However, arguing for our model, with wild type proFP2-GFP, and more often with wild type proFP3-GFP, some localization in cytostome-like vesicular structures on the plasma membrane was seen in young parasites. Although fur-
ther study will be needed to better characterize the architecture of these membrane structures, our results support the notion that the plasma membrane is an intermediary site of protein trafficking to the food vacuole. However, this route is apparently not taken by all food vacuolar proteins, as a recent study revealed that a newly identified food vacuolar protein, FCP (FVYE domain-containing protein), is trafficked to the food vacuole directly from the cytoplasm (30).

As seen earlier for a variety of GFP fusion proteins in P. falciparum (23, 31–33), in addition to full-length fusion protein, we observed processed GFP in parasite extracts (supplemental Fig. S1). The cleavage of GFP was more pronounced in constructs that were localized to the food vacuole, suggesting that some GFP processing occurred in this organelle. GFP cleavage might lead to fluorescence at sites of transport of exclusively free GFP. However, localization of both GFP and native falcipain-2 in the food vacuole with a full prolafcinai-2 construct (Fig. 1) and differential trafficking of other constructs, with many showing little food vacuole fluorescence, argue strongly that fluorescence was indicative of localization of falcipain-2 constructs and thus informative regarding trafficking determinants for the enzyme.

We observed that mutant GFP chimeras that were arrested at the plasma membrane during the trophozoite stage of the life cycle were found in the food vacuole in mature schizonts. Our results suggest that trafficking to the food vacuole in late schizonts was via a default pathway that likely traffics many proteins to this organelle for hydrolysis prior to completion of the erythrocytic cycle. Importantly, it is the trafficking of falcipains in trophozoites and early schizonts that is most relevant for parasite metabolism, as expression of falcipain-2 and falcipain-3 and hemoglobin hydrolysis occur primarily at these stages (11, 12). Similarly, some (presumably default) trafficking to the food vacuole has also been seen with PEX1, a parasitophorous vacuolar protein (31), and PfEMP1, a protein targeted to the erythrocyte plasma membrane (33).

The trafficking constructs evaluated in this study included a number with an unusual phenotype, with localization in a patchy configuration apparently contiguous with the plasma membrane. This phenotype was distinct from that of other constructs that demonstrated diffuse membrane localization. This result suggests that the P. falciparum plasma membrane may have a more complex architecture than is currently appreciated, with accumulation of trafficking intermediates at discrete sites, perhaps overlapping with cytoskeletons that are taking up erythrocytic contents or with ports that mediate cargo-specific endocytosis. It is also possible that proteins are held in these structures by cytoskeletal molecules. Interestingly, this localization pattern requires an intact C terminus of the prodomain, as this phenotype was seen only in point mutants and internal deletion constructs, whereas truncated fusion proteins distributed uniformly through the plasma membrane. Therefore, it is possible that the C-terminal portion of the prodomain might interact with proteins that anchor falcipains to patches on the membrane as a prerequisite to transport to the food vacuole. In any event, our results suggest that molecules that inhibit falcipain trafficking might inhibit parasite development by blocking hemoglobin hydrolysis, as is the case with inhibitors of the enzymatic functions of falcipain-2 and falcipain-3 (7). If targeting signals or pathways are shared by other proteins, disruption of trafficking may engender marked antiparasitic effects. Thus, further exploration of mechanisms by which proteins are trafficked to the P. falciparum food vacuole may identify targets for novel means of antimalarial chemotherapy.

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