Traffic to the Malaria Parasite Food Vacuole

A NOVEL PATHWAY INVOLVING A PHOSPHATIDYLINOSITOL 3-PHOSPHATE-BINDING PROTEIN

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Phosphatidylinositol 3-phosphate (PI3P) is a key ligand for recruitment of endosomal regulatory proteins in higher eukaryotes. Subsets of these endosomal proteins possess a highly selective PI3P binding zinc finger motif belonging to the FYVE domain family. We have identified a single FYVE domain-containing protein in Plasmodium falciparum which we term FCP. Expression and mutagenesis studies demonstrate that key residues are involved in specific binding to PI3P. In contrast to FYVE proteins in other organisms, endogenous FCP localizes to a lysosomal compartment, the malaria parasite food vacuole (FV), rather than to cytoplasmic endocytic organelles. Transfections of deletion mutants further indicate that FCP is essential for trophozoite and FV maturation and that it traffics to the FV via a novel constitutive cytoplasmic to vacuole targeting pathway. This newly discovered pathway excludes the secretory pathway and is directed by a C-terminal 44-amino acid peptide domain. We conclude that an FYVE protein that might be expected to participate in vesicle targeting in the parasite cytosol instead has a vital and functional role in the malaria parasite FV.

Malaria parasites inflict a tremendous global burden on the health and productivity of human kind. Among the species predominantly infective to humans, Plasmodium falciparum stands out as responsible for more than half of all infections and for causing the most severe forms of the disease resulting in the death of more than 1.5 million people annually (1). Malaria parasites circulate in the human host by repeated invasion and development within the human erythrocyte. During the 48-h cycle of development, parasites endocytose 80–90% of the host cell cytosol and hemoglobin. Specialized proteases are believed to traffic to vesicles containing hemoglobin to initiate the digestion of hemoglobin on route to its final destination, the lysosomal compartment, or parasite food vacuole (FV)6 (2). Although the endocytic process is characterized by morphologically distinct parasite structures including specialized structures for the uptake of hemoglobin (cytostomes), hemoglobin-containing vesicles, and a single FV (3), the contribution of protein components to the endocytic pathway required for membrane trafficking, molecular signaling, effector protein recruitment, and formation of these structures remains unknown.

Phosphatidylinositol 3-phosphate (PI3P) plays a fundamental regulatory role in endocytic systems of higher eukaryotes (4–6). Rab5, a universally conserved component of early endosomes, recruits the type III PI 3-kinase in mammals and yeast. Hence, the product of this kinase, PI3P, is found in the early endosomes of mammals and is likewise restricted to the endocytic pathway in yeast (6–8). Various other endosomal regulatory proteins bind PI3P and thereby localize to endocytic compartments in yeast and mammals (9). Many among these including the early endosomal autoantigen-1 (EEA1) contain a highly selective PI3P binding zinc finger motif belonging to the FYVE domain family (conserved in Fab1, YOTB, Vac1, and EEA1) (9).

Although P. falciparum appears limited with regard to Rab5 effectors and other endocytic machinery, it does possess a phosphatidylinositol biosynthetic pathway (10). We, therefore, asked if P. falciparum possesses functional FYVE domain proteins. Using a bioinformatics approach we identified a single FYVE domain-containing protein encoded in the parasite genome. Surprisingly, this protein did not localize to endocytic structures as would be predicted for an FYVE domain family protein but, rather, localized discretely to the lumen of the FV, a lysosomal compartment characterized by the presence of hemozoin (a crystallized heme byproduct of hemoglobin digestion). By identifying the domain responsible for targeting to the FV, we not only explain this unexpected result but identify a new direct trafficking pathway between the parasite cytosol and the parasite FV.

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6 The abbreviations used are: FV, food vacuole; EEA1, early endosomal autoantigen-1; AD, activation domain; BD, DNA binding domain; ER, endoplasmic reticulum; BFA, brefeldin A; Cvt, cytoplasmic to vacuole trafficking; TGN, trans-Golgi network; HRPII, histidine-rich protein II; PI3P, phosphatidylinositol (PI) 3-phosphate; GFP, green fluorescent protein.
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EXPERIMENTAL PROCEDURES

In Vitro Cultivation of P. falciparum—P. falciparum strain 3D7 (obtained from MR4) was employed in experiments contributing to Figs. 4 and 5, whereas strain FCR3 (a gift from Dr. Kasturi Haldar) was used to obtain Figs. 6–9. Parasites were maintained in 5% CO₂ (for FCR3) or 90% N₂, 5% CO₂, and 5% O₂ for (3D7) in leukocyte-free erythrocytes of blood group A+ (American Red Cross) at 5% hematocrit and cultured in RPMI 1640 medium (Invitrogen) supplemented with 25 mM HEPES, sodium bicarbonate (2 g liter⁻¹), gentamicin (1 μg ml⁻¹), 92 μM hypoxanthine and containing 10% human serum, type AB as described (11). Parasite synchronization was achieved by isolation of late stage trophozoites on Percoll sorbitol gradients as described (12) followed by reintroduction into culture with fresh red blood cells. Four hours after the first detection of ring stage parasites, cultures were treated with 5% sorbitol (13) to lyse all remaining late stage parasites. This yielded highly synchronous cultures.

Cloning of FCP and Plasmid Constructs—Using open reading frame-specific primers with appropriate 5‘-end restriction sites, FCP cDNA was reverse transcription-PCR amplified from total asexual stage P. falciparum RNA using RT-Superscript II (Invitrogen) and KOD hot start DNA polymerase (Novagen Inc.). For yeast 2-hybrid analysis, FCP cDNA was appropriately digested and ligated into yeast two-hybrid vectors pACT-2 and pGBT-9 (Invitrogen) between the BamHI and XhoI sites of pACT-2 and EcoRI and BamHI sites of pGBT-9. Ligation were made in-frame and downstream of the GAL4 activation domain (AD) or DNA binding domain (BD) to obtain pAD-FCP and pBD-FCP. The cDNA sequence of cloned FCP was identical to that of the putative gene (Pf14_0574) represented in the 3D7 P. falciparum genome data base.

For GFP expression studies in Plasmodium, the eGFP gene of pEGFP-C2 (Invitrogen) was replaced with the GFP-M2 gene of pHHDGFP (14). This yielded the plasmid pGFP-M2-C2. Subsequently, GFP-M2 was subcloned from the yeast expression vector pBD-FCP into the EcoRI and Sall sites of pGFP-M2-C2 such that the gene fusion was in-frame and downstream of GFP-M2. Domain deletions were constructed from pBD-FCP and converted to GFP-M2 gene fusions by ligation between the EcoRI and Sall sites of pGFP-M2-C2. GFP-M2 was obtained by digestion of pBD-FCP with BglII and BamHI followed by blunt ending and self-ligation. This removed the entire C terminus including the complete coiled-coil domain. GFP-M2 was obtained by digestion of pBD-FCP with KpnI and BglII followed by blunt ending and self-ligation. This deleted only the FYVE domain and kept the N terminus and remainder of GFP in-frame. GFP-M2 was obtained by digestion of pBD-FCP with Styl and BamHI followed by blunt ending and self-ligation. This removed the C-terminal conserved domain encoding the final 44 amino acids of GFP. Transfer to pGFP-M2-C2 yielded the following plasmid constructs: pGFP-M2-C, pGFP-M2-FYVE, and pGFP-M2-c44. To explore the function of the C-terminal 44 amino acids, pGFP-M2 was constructed by PCR amplification of the C-terminal 132 bp encoding the 44-amino acid peptide using forward and reverse primers bearing EcoRI and Sall sites, respectively, and cloning into the EcoRI and Sall sites of pGFP-M2-C2. Primers (GFP-M2-ATTB1, 5′-GGGGA-CAGGTGTGACACAAAATGAGTAA-AGGAGAAGACTTTTTC-3′, and GFP-M2-ATTB2, 5′-GGGGA-CCATTGTGACAAGAAGCTGGTTGACTGTTACATC-TAGATCCCG-3′) flanking the GFP gene fusion cassettes in the above plasmids, which contained 5′ attB1 and attB2 recombination sites, respectively, were used to PCR amplify all GFP-gene fusions such that they were suitable for cloning into pDONR-21 (Invitrogen) using the BP reaction and Gateway™ recombination cloning system (Invitrogen). Subsequent LR reactions (Invitrogen) with the Plasmodium Gateway™ destination vector pH11-DR0.28-DEST (15) (a kind gift from Prof. Tina Skinner-Adams, Queensland Institute of Medical Research, Australia) resulted in the following Plasmodium GFP expression vectors: EXP- GFP-M2, EXP-GFP-FCP, EXP-GFP-FCPDΔ-c-coil, EXP-GFP-FCPΔ-c44, EXP-GFP-FCPΔ-FYVE, EXP-GFP-44aa. These expression vectors all utilize the Plasmodium HSP86 5′-untranslated region as a constitutive promoter for the expression of the GFP gene fusions.

For protein expression studies, FCP cDNA was cloned in PQE-UA vector (Qiagen) to facilitate its expression as an N-terminal His₆-tagged protein. Site-directed mutagenesis was performed by using the QuikChange kit (Stratagene).

Recombinant Protein Expression in E. coli and Anti-FCP Antiserum Production—BL21-RIL E. coli (Stratagene) was transformed with PQE-FCP plasmid or its variants. Cultures were grown in LB media containing 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Recombinant protein was induced at mid-logarithmic phase (A₆₀₀ value of 0.6) by the addition of 1 mM isopropyl-1-thio-D-galactopyranoside either at 37 °C for 4 h or at 18 °C for 14 h. Bacterial cells were harvested by centrifugation at 4000 × g for 30 min and suspended in buffer A (50 mM Tris, pH 7.4, 6 mM NaCl, 300 mM imidazole, 1 mM dithiothreitol, and 6 mM urea). Eluted proteins were renatured by dialysis against buffer containing reduced amounts (4 to 0 mM) of urea. Refolded recombinant proteins were analyzed by gel filtration and SDS-PAGE, and a protein band of the expected molecular mass of 37 kDa was detected by Coomassie staining. To confirm the identity of the recombinant protein, the 37-kDa protein band was excised and subjected to tryptic digest and mass spectrometric analysis by the Institute of Molecular Medicine, The Chatterjee Group, New Delhi (supplemental Fig. S1). To raise anti-sera against FCP, 100 μg of recombinant FCP was emulsified with complete Freund’s adjuvant and used to raise antisera in mice and rabbits.

Dot-blot Phosphoinositide Binding Assays—Dot-blot assays were performed as described previously (16). Briefly, various phosphoinositides were serially diluted and spotted on nitrocellulose membrane and air-dried for 2 h. Subsequently, the membrane was blocked with 3% bovine serum albumin in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 0.1% Tween 20) for 3 h. The membrane was incubated with recombinant His₆-FCP (0.5 μg/ml) or its variants diluted in...
blocking buffer for 12 h at 4 °C. The membrane was washed 5 times with buffer A before 3 h of incubation with anti-His$_6$ antibody (BD Biosciences). Subsequently, the membrane was incubated with horseradish peroxidase-labeled anti-mouse IgG, and phosphatidylinositol-bound protein was detected by chemiluminescence.

**Yeast Two-hybrid Growth Assays**—Yeast two-hybrid liquid culture growth assays were performed as previously described (17). Yeast were transformed with all possible paired plasmid combinations as well as with individual plasmids as controls, and protein-protein interactions were determined based on growth in His-dropout medium. Stringency was further assessed in increasing concentrations of 3-amino-1,2,4-triazol.

**Immunoblotting and Immunolocalization**—For immunoblot experiments described in Fig. 5, parasite material was separated from erythrocytes and parasitophorous vacuole-derived material by lysis in 0.1% saponin analogous to a previously published procedure (33). An equal number of parasites fractionated into a pellet or concentrated supernatant was denatured by boiling in 2% SDS, applied to SDS-PAGE, and subsequently transferred to nitrocellulose. Immunoblotting was performed using rabbit anti-FCP and horseradish peroxidase-labeled goat anti-rabbit IgG. Blots were developed using a WestDura ECL kit (GE Healthcare). For immunofluorescence, parasitized red blood cells were spread on slides and fixed in methanol for 10 min. Subsequently, slides were blocked with 3% bovine serum albumin for 12 h at 4 °C and incubated with a 1:50 dilution of mouse anti-FCP antisera. After washing with phosphate-buffered saline, slides were stained with goat anti-mouse IgG conjugated to Texas Red. Cells were visualized on a Zeiss LSM510 confocal microscope equipped with a CCD camera. Control experiments were performed using preimmune bleeds.

For immunoelectron microscopy, parasites were fixed in 4% paraformaldehyde (EM Polysciences Inc.), 0.1% glutaraldehyde (EM Polysciences Inc.), and 0.25 M HEPES, pH 7.4, embedded in 1:1 phosphate-buffered saline containing 10% bovine skin gelatin (Sigma) and incubated at 4 °C in cryo-protectant containing 10% polyvinylpyrrolidone and 2.7 M sucrose. Subsequently, parasite blocks were applied to pins, snap-frozen in liquid nitrogen, and subjected to ultra thin sectioning in a liquid nitrogen-cooled cryo-ultramicrotome. Sections were applied to nickel-coated grids, incubated with a 1:1000 dilution of affinity-purified rabbit anti-FCP IgG at 25 °C, and stained with gold-conjugated goat anti-rabbit IgG. Electron micrographs were obtained digitally by a CCD camera using a Philips Techni BioTwin electron microscope.

**Parasite Transfection, Localization, and Dominant Negative Effects**—Transient transfections of malaria parasites with EXP-GFP vectors (see above details) were performed by a modification of the previously described preloading technique (18). 200 μl of packed red blood cells were washed once in incomplete cytostix (19) containing 120 mM KCl, 0.15 mM CaCl$_2$, 2 mM EGTA, 5 mM MgCl$_2$, 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$, and 25 mM Heps, pH 7.6) and then resuspended to 400 μl in ice-cold incomplete cytostix. Red blood cells were then preloaded with 50 μg of plasmid DNA by electroporation in a ECM630 Electro Cellular Manipulator™ (BTX) at settings of 310 V, 25 ohms, and 950 microfarads. To ensure synchronous transfections and facilitate morphological interpretation of potential dominant negative effects, cultures were synchronized as described above. Subsequently, late stage parasites were once again purified by Percoll sorbitol gradient as described above and then added to preloaded red blood cells and cultured for 40–46 h before microscopic analysis for GFP expression and morphological detail. Microscopy was performed at 1000× magnification using a Nikon Optiphot II microscope equipped with epifluorescence and Nomarski optics or at 1000× using a Zeiss Axioscope with epifluorescence. FV localization was verified by photography of cells under simultaneous fluorescence and bright field phase contrast at low light levels. Images were collected by CCD camera, labeled in Adobe PhotoShop 6.0 (Adobe), and arranged in Adobe Illustrator 10 (Adobe).

**RESULTS**

*Plasmodium Encodes a Single FYVE Domain Protein*—Sequence alignment of FYVE domains yielded the consensus sequence R(R/K)HHCR, which was used to search the *P. falciparum* genome data base. This revealed only a single FYVE domain-containing protein (PF14_0574). Analysis by SOCKET (20) indicated the presence of a coiled-coil domain (residues 163–266) downstream of the FYVE zinc finger domain (residues 42–92). Thus, we named the protein FCP for FYVE and coiled-coil domain-containing protein. An additional gene (PF13_0055), annotated as encoding a potential FYVE domain, more closely resembled a RING domain and, hence, was not investigated further.

Sequencing of FCP cDNA confirmed a lack of introns, and comparison of the amino acid sequence with other orthologous counterparts indicated that FCP was highly conserved with 70–80% amino acid identity within the malaria parasite genus *Plasmodium* (Fig. 1A). Additional searches indicated that at least two potential FYVE domain-containing proteins exist in the related parasite *Toxoplasma gondii*. TBLASTN searches excluding the FYVE domains of other family members including EEA1, Hrs, YOT1, Fab1, and Vac1 failed to identify genes encoding proteins of significant homology in *P. falciparum*. This provided the first indication that FCP might not function in the same fashion as the endosomal FYVE proteins of higher eukaryotes.

X-ray crystal structures for Saccharomyces cerevisiae Vps27p, Drosophila melanogaster Hrs, and human EEA1 have further defined the secondary and tertiary structure of the FYVE domain as containing a core fold similar to that of the RING domain (21). FCP encodes all of the conserved residues and attributes known to be required for PI3P binding by other FYVE domain proteins. Among these are eight invariant zinc-coordinating cysteine residues arranged in four CXXC motifs, an upstream WXXD motif, and a downstream RVC motif, each intimately involved in PI3P binding (Fig. 1B). In addition, the glycine residue immediately adjacent to the second pair (CXXC) of zinc-coordinating cysteines is invariant among all known FYVE domain proteins and is believed to provide flexibility required for formation of the core fold of the domain (Fig. 1B). The R(R/K)HHCR consensus sequence is known to be the principal site of PI3P binding (Fig. 1B).

**FCP Specifically Interacts with PI3P**—To better understand phosphoinositide interactions with FCP, we performed lipid
dot-blot assays using recombinant wild type and site-directed mutants of FCP. Wild type FCP bound specifically to PI3P (Fig. 2B). Deletion of the FYVE domain (data not shown) resulted in a complete loss of phosphoinositide binding as did a site-directed substitution K55A (Fig. 2B), which resides within the conserved motif R(R/K)HHCR (Figs. 1A and 2A). The crystal structure of the EEA1 FYVE domain bound to inositol 1,3-diphosphate has revealed that amino acid residues form a coordination network with the 1\(^\text{st}\) and 3\(^\text{rd}\) phosphates of PI3P, and it has been hypothesized that these residues may lend specificity to FYVE-PI3P interaction (22). To test this we replaced all flanking arginines (Arg-52, Arg-54, and Arg-59) with neutral alanine residues. This three-amino acid substitution resulted in a loss in specificity for PI3P as indicated by increased binding to phosphoinositides, PI 3,4,5-triphosphate and PI 4,5-diphosphate. This loss in PI3P binding specificity demonstrated that indeed a critical interaction does exist between these basic residues and the 1\(^\text{st}\) and 3\(^\text{rd}\) phosphates of PI3P (Figs. 2, A and B).

**FCP Self-associates**—Several FYVE proteins have been shown to dimerize by virtue of a coiled-coil domain, thereby increasing their avidity for PI3P (23, 24). For example, EEA1 dimerizes when bound by Rab5 and then simultaneously binds two PI3P molecules in the early endosome (25). In this way EEA1 is believed to participate in tethering of opposing vesicu-
The interaction between AD-human growth curve from yeast expressing a positive control interacting and BD vector only, enhanced growth demonstrating a modulation of yeast expressing a negative control pair, AD-FCP respectively. A representative plot (Fig. 3) shows inhibited growth of yeast expressing a negative control pair, AD-FCP and BD vector only, enhanced growth demonstrating a modest interaction between AD-FCP and BD-FCP and a robust growth curve from yeast expressing a positive control interaction between AD-human μ1 and BD-trans Golgi network-binding protein (BD-TGN) (17).

Results from this analysis suggested an interaction between AD-FCP and BD-FCP. When recombinant FCP was chromatographed on a Superdex-75 gel filtration column, it too exhibited dimerization or self-association (Fig. 3B). The strong tendency of FCP to dimerize was also indicated by the presence of a 74-kDa dimeric species on an incomplete denaturing SDS-PAGE gel, which was confirmed by Western blot and mass spectrometric analyses to be FCP (supplemental Fig. S1). We, therefore, conclude that FCP, like other FYVE domain proteins, can undergo a self-association. Given the involvement of coiled-coil domains in the oligomerization of other proteins, it is likely that dimerization of FCP is mediated by the coiled-coil domain of FCP. This dimerization event is likely to increase the avidity of the protein-lipid interaction by coordinating the presentation of two FYVE domains in tandem to two available PI3P ligands instead of one (27).

**FCP Traffics to the Lumen of the FV in Late Trophozoites**—Western blot analyses of stage-specific *P. falciparum* revealed that expression of FCP occurs during the late trophozoite stage of the parasite intra-erythrocytic life cycle (Figs. 4, A and B). Although this stage is known to be the most active stage for consumption of host cell hemoglobin and metabolism, FCP was remarkably not found to be localized to cytoplasmic endocytic vesicles or hemoglobin uptake structures but rather to the parasite FV, a lysosomal compartment characterized by the presence of hemozoin crystals (Fig. 5A).

To determine whether FCP traffics to the outer surface, inner surface or lumen of the FV, cryo-immunoelectron microscopy using rabbit anti-FCP was employed. As shown in Figs. 6, A and B, immunogold particles specifically stained the more electron lucent lumen of the FV. Fig. 6A shows a close-up of a cross-section through a folded FV with immunogold staining for FCP most abundant in the electron lucent lumen and to a lesser extent in association with hemozoin. Less than 1% of background staining was observed outside of the FV, evident in Fig. 6B, which shows the cross-section of a host cell, parasite, and its FV. Likewise, no significant immunogold staining was observed on other host cell or parasite structures. As evidenced in Fig. 6C, which shows three well-defined parasites, each lacking a cross-section through a FV. These observations indicate that FCP traffics and translocates to the lumen of the parasite FV in late trophozoites and not merely to the surface.

**FCP Traffics via a Mechanism That Excludes the Classical and Alternate Secretory Pathways**—Sorting mechanisms for targeting proteins to the lysosomes of higher eukaryotes principally begin with entry of the proteins into the secretory pathway. Lysosomal proteins are synthesized and enter the rough ER and proceed to the Golgi where they are directed by recep-
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A. anti-FCP and anti-FCP + BFA

B. anti-HRPII and anti-HRPII + BFA

C. FCP with Saponin (Total, Pellet, Sup)

FIGURE 5. FCP localizes to the FV but does not traffic via the classical or alternate secretory pathways. A, immunofluorescence using anti-FCP revealed a discrete localization to the parasite FV. Ring stage parasites were treated either with 5 μM BFA (anti-FCP + BFA) or solvent as control (anti-FCP) for 12 h before immunofluorescence microscopy. BFA treatment did not alter FV localization of FCP. B, as a control experiment, parasites treated with BFA (anti-HRPII + BFA) or without BFA (anti-HRPII) were stained using antisera against a known secreted protein, HRPII. HRPII was localized predominantly to the cytoplasm of the host infected erythrocyte (anti-HRPII). BFA treatment resulted in a significant loss in erythrocyte localization of HRPII and a concomitant increase in the parasite cytoplasm (anti-HRPII + BFA). C, trophozoite-infected erythrocytes were treated with saponin to separate the erythrocyte and parasitophorous vacuole contents (Sup) from the parasite material (Pellet), and immunoblotting was performed for FCP. FCP was found in the pellet fraction rather than the concentrated supernatant fraction.

As a protease, plasmepsin II has been shown to be secreted before endocytosis and trafficking to the FV (2); we needed to further confirm that a transient or rapid secretion event to either the parasitophorous vacuole or red blood cell cytoplasm did not occur. To address this possibility, parasitized red blood cells were treated with 0.1% saponin (33). This treatment is known to permeabilize both erythrocyte and parasitophorous vacuole membranes (13, 33). FCP was not found in the concentrated supernatant fraction but remained in the pellet or parasite soma fraction (Fig. 5C), indicating its absence from the secretory pathway. This further suggests that FCP may circumvent the secretory pathways and instead indicates that a novel trafficking pathway is at work.

The FYVE Domain Does Not Affect Trafficking of FCP but Is Functionally Critical to the Parasite—As seen for the endogenous protein (Figs. 5 and 6), transient transfection and expression of GFP-FCP in P. falciparum revealed a discrete localization of the tagged protein to the FV (Fig. 7A). To further delineate functional attributes of the conserved domains of FCP and to identify the domains responsible for the targeting, we transfected parasites with plasmid constructs containing various domain deletions of FCP fused to the C terminus of GFP (Fig. 7B). Transient transfection and live cell fluorescence were employed throughout the transfection studies to avoid artifacts due to selection of clonal transfectant populations or fixation artifacts that may alter morphology and fluorescence patterns. Transfectants expressing a deletion of the FYVE domain (GFP-FCP-ΔFYVE) displayed a discrete spot of fluorescence (Fig. 7C) that we later show coincides with the parasite FV (see Fig. 9A). This pattern indicated that PI3P binding is not involved in the targeting event. Of note, the dwarfed morphologies of the GFP-FCP-ΔFYVE transfectants appeared to be the result of a dominant negative growth defect. Parasites failed to attain the same size as normal 30–36-h trophozoites and also failed to develop large hemozoin crystals or FVs with wild type morphology and fluorescence patterns. Transfectants expressing a deletion of the FYVE domain (GFP-FCP-ΔFYVE) displayed a discrete spot of fluorescence (Fig. 7C) that we later show coincides with the parasite FV (see Fig. 9A). This pattern indicated that PI3P binding is not involved in the targeting event. Of note, the dwarfed morphologies of the GFP-FCP-ΔFYVE transfectants appeared to be the result of a dominant negative growth defect. Parasites failed to attain the same size as normal 30–36-h trophozoites and also failed to develop large hemozoin crystals or FVs with wild type dimensions. A more detailed investigation of this mutant is discussed below (see Fig. 9).

Deletion of the C terminus including deletion of the coiled-coil domain (GFP-FCP-Δ-c-coil), while retaining the FYVE domain, ablated trafficking of GFP to the FV as seen by the cytoplasmic fluorescence pattern (Fig. 7C). This, once again demonstrated that the PI3P binding domain is not involved in targeting FCP to the FV. From the above findings we concluded...
that deletion of the FYVE domain generates a dominant negative phenotype, which likely impairs hemoglobin processing and/or uptake but not trafficking.

The C-terminal 44-Amino Acid Domain of FCP Is Necessary and Sufficient to Target FCP to the FV—To further delineate which regions in the C terminus mediate targeting, we deleted the conserved C-terminal 44 amino acids (GFP-FCP-Δc44) downstream of the coiled-coil domain. As can be seen from the cytoplasmic fluorescence pattern (Fig. 7C), targeting to the FV was once again ablated, indicating that this C-terminal peptide is indeed required for trafficking GFP to the FV (Fig. 7C).

To determine whether the C-terminal 44 amino acids are sufficient for targeting to the FV, we tagged the C terminus of GFP with the 44-amino acid peptide, thus eliminating all of the other potentially confounding components of FCP. Indeed, transfection of GFP-44aa resulted in trafficking of GFP to the FV (Fig. 8A), whereas transfection with GFP control vector alone resulted in a cytoplasmic fluorescence that notably excluded the FV (Fig. 8B). It is, thus, evident that the 44-amino acid peptide, notably conserved between diverse rodent and primate species of Plasmodium, functions as a direct cytoplasmic to vacuole trafficking (Cvt) signal.

Dominant Negative Effects of the FYVE Deletion Mutant—As mentioned above, deletion of the FYVE domain revealed a dominant negative growth or developmental defect but did not appear to alter localization to the FV (Fig. 7C). The GFP-FCP-ΔFYVE-expressing parasites failed to mature morphologically into normal trophozoites. Given their small size and diminutive FVs, morphologies of mutant parasites were poor, making clear localization of signal to the FV difficult.

**FIGURE 6.** FCP localizes to the lumen of the FV. **A**, cryo-immunoelectron micrograph showing a close-up of a folded FV with immunogold-stained FCP in the lumen of the parasite FV in a late trophozoite. Various structures are indicated as host red blood cell cytosol (RBC Cytosol), parasite body (parasite), parasite nucleus (Nu), FV, and hemozoin (Hz). **B** shows a more complete parasite with a cross-section through the FV indicating a lack of immunogold staining outside the FV. **C**, three parasites from a different region of the same ultrathin section that was immunogold-stained for anti-FCP in **A** and **B**. Note the lack of concentrated gold particles on all visible parasite structures including ER, parasite plasma membrane (PPM), parasitophorous vacuolar membrane (PVM), and hemoglobin containing vesicles (HV). As apparent from **A** and **C**, less than 1% of immunogold appeared outside of the parasite FV.
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Regardless of the altered morphologies, expression of GFP-FCP-ΔFYVE yielded a GFP fluorescence pattern that coincided with visible, albeit diminutive FVs within stunted parasites (Fig. 9A) as compared with untransfected parasites in the same culture (data not shown) and 36-h parasites expressing a GFP control vector (Fig. 9B).

For P. falciparum, ring stage parasites represent the first 24 h of development within the red blood cells. Trophozoites represent the next 16 h of development, and finally, schizonts, characterized by 8–32 distinct nuclei, represent the last 8 h of development within red blood cells. Because trophozoites are morphologically characterized by the presence of a visible FV-containing hemoglobin, the 36-h GFP-FCP-ΔFYVE-expressing parasites we observed (Fig. 9A) were representative of morphologically stunted trophozoites with one or more defects in hemoglobin uptake or metabolism.

To further confirm that GFP-FCP-ΔFYVEs were not developmentally arrested rings but rather stunted or defective trophozoites, we harvested parasites at 36 h post-transfection by centrifugation through Percoll-sorbitol gradients as described previously (12). This method is based on density differences triggered by parasite-induced new permeation pathways that result in the increased solute permeability of host red blood cells infected with later stage parasites (trophozoites and schizonts) as compared with early stage parasites (rings) (12). Because the GFP-FCP-ΔFYVE-expressing parasites were isolated in the trophozoite fraction of Percoll-sorbitol gradients (data not shown), we believe that they were developmentally mature enough to display new permeation pathways.

DISCUSSION

A variety of signal sequence topologies are defined in P. falciparum that mediate targeting to membranes or organelles inside or outside of the parasite. Conserved conventions such as N-terminal mitochondrial targeting signals are known to be functional in Plasmodium (34, 35). In addition, the presence of a chloroplast remnant organelle termed the apicoplast adds additional complexity to the trafficking of proteins in malaria. Trafficking to the apicoplast has been found to use an N-terminal pre-sequence (36, 37). This bipartite sorting signal consists of an N-terminal signal peptide for entry into the secretory pathway via the ER followed by an apicoplast membrane transit peptide that permits transit across the membranes of the apicoplast (37). Similarly for secretion, a tripartite sorting signal consisting of an N-terminal signal sequence followed by two transit peptides directs secretion of proteins from the parasite across the parasite plasma membrane and surrounding parasitophorous vacuolar membrane for delivery into the host red blood cell (29, 30, 38). A limited number of secreted proteins with a conventional N-terminal signal sequence are targeted to the parasitophorous vacuolar membrane or other membranes (e.g. EXP-1). The aspartic proteases plasmepsins I and II are type II integral membrane proteins with internal signal sequences and are thought to be delivered to the parasite FV initially via the secretory pathway, which later converges with the parasite endocytotic pathway (2). In a similar fashion the principal sorting mechanisms for targeting proteases and hydrolases to the lysosomes of mammals and yeast include entry into the ER and secretory pathway followed by receptor-mediated vesicular transport initiated in the trans-Golgi, which redirects secretory...
that excluded the FV (yeast has been proposed for two soluble proteins, the pro-form direct receptor-mediated transport to the digestive vacuole of autophagy pathway, have emerged from studies on reviewed in Huang and Klionsky (39). This less traveled path of exists perhaps for FV proteins that lack N-terminal or internal signal sequences typically required for entry into the secretory pathway, and neither contains a transmembrane span. In addition and as seen above in Fig. 5, treatment with BFA, known to block both the classical and alternate secretory pathways in Plasmodium, fails to alter trafficking of FCP to the FV. Hence, our data suggests that a non-secretory Cvt pathway exists perhaps for FV proteins that lack N-terminal or internal signal sequences.

Precedents for a Cvt pathway, also known as a constitutive autophagy pathway, have emerged from studies on S. cerevisiae, reviewed in Huang and Klionsky (39). This less traveled path of direct receptor-mediated transport to the digestive vacuole of yeast has been proposed for two soluble proteins, the pro-form of the hydrolyase API (40, 41) and the α-mannosidase I precursor (Ams1p) (42, 43). Like FCP, these examples lack N-terminal signal sequences, do not enter the secretory pathway, and have peptide signals that are necessary for directed targeting to the yeast digestive vacuole. Indeed, the extreme C terminus of Ams1p has also been fused to the normally secreted protein, invertase, resulting in redirection of the chimeric invertase from the secretory pathway to the yeast vacuole (42). Disruption of these trafficking signals results in a redistribution of the hydrolyses to the cytosol instead of the lysosome as we have observed for GFP-FCP variants lacking the C-terminal trafficking signal (41, 42). As with these examples in yeast, at present there is no obvious primary sequence similarity shared between the FCP trafficking peptide and other known FV proteins found in Plasmodium.

A link to PI3P functions in Cvt as well as in endocytosis has emerged in a study of Etf1, another PI3P-binding protein from yeast. Etf1 was identified as an effector of the yeast PI 3-kinase (Vps34), and deletion of Etf1 or mutation of its PI3P binding domain causes a severe defect only in Cvt/constitutive autophagy, not in endocytosis (44). In this regard Vps34 and its product PI3P are known to be involved in endocytosis, classical sorting of hydrolases such as CPY to the lysosome, starvation induced macroautophagy, and Cvt. By contrast, ETF1 appears only to be involved in the Cvt pathway (44).

Our domain analysis identifies and reveals that different functional attributes (PI3P binding, oligomerization, and FV trafficking) exist in modular domains of FCP. Conservation of FCP throughout the genus Plasmodium and an apparent lack of other FYVE domain-containing proteins in Plasmodium argue in favor of a vital non-redundant function for FCP in malaria parasites. The dominant negative effects of the FYVE domain deletion construct further argue for a vital function. In particular, the negligible amount of hemozoin in the ΔFYVE mutant parasites suggests a functional contribution by FCP either directly or indirectly to the metabolism of hemoglobin, general function of the FV, or polymerization of heme. Indeed, the FV provides vital functions to the parasite (45), and several antimalarial compounds are thought to act by inhibition of heme polymerization (46). A better understanding of protein and lipid trafficking mechanisms and characterization of FV resident proteins such as FCP should aid in the development of strategies to combat malaria.

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