Protein Targeting to the Parasitophorous Vacuole Membrane of *Plasmodium falciparum*† ‡

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Received 15 January 2011/Accepted 31 March 2011

Red blood cell (RBC) invasion and parasitophorous vacuole (PV) formation by *Plasmodium falciparum* are critical for the development and pathogenesis of malaria, a continuing global health problem. Expansion of the PV membrane (PVM) during growth is orchestrated by the parasite. This is particularly important in mature RBCs, which lack internal organelles and no longer actively synthesize membranes. Pf16, a 16-kDa integral PVM protein expressed by gametocytes, was chosen as a model for studying the trafficking of material from the parasite across the PV space to the PVM. The locations of Pf16-green fluorescent protein (GFP) reporter proteins containing distinct regions of Pf16 were tracked from RBC invasion to emergence. Inclusion of the 53 C-terminal amino acids (aa) of Pf16 to a GFP reporter construct already containing the N-terminal secretory signal sequence was sufficient for targeting to and retention on the PVM. An amino acid motif identified in this region was also found in seven other known PVM proteins. Removal of the 11 C-terminal aa did not affect PVM targeting, but membrane retention was decreased. Additionally, during emergence from the PVM and RBC, native Pf16 and the full-length Pf16-GFP reporter protein were found to concentrate on the ends of the gametocyte. Capping was not observed in constructs lacking the amino acids between the N-terminal secretory signal sequence and the transmembrane domain, suggesting that this region, which is not required for PVM targeting, is involved in capping. This is the first report to define the amino acid domains required for targeting to the *P. falciparum* PVM.

A malaria infection begins with sporozoites are introduced by the bite of an infectious mosquito. The sporozoites must then actively invade liver cells by inducing an invagination of the liver cell plasma membrane to form a parasitophorous vacuole (PV) (25). Once the invading sporozoite is enclosed in the PV, a developmental cascade is triggered that leads to the formation of tens of thousands of merozoites in 5 to 7 days. The merozoites are then released from the liver cell and actively invade red blood cells (RBCs), again forming a parasitophorous vacuole from the RBC plasma membrane as they enter (6). Once inside the PV in the RBC, the parasite can either multiply asexually, producing 16 to 32 new merozoites to continue the infection in the host, or initiate sexual differentiation to produce a single male or female gametocyte (1). Gametocytes are required to transmit the infection to the mosquito and consequently spread the infection to other humans.

During invasion, the lipids that make up the PV membrane (PVM) are largely derived from the plasma membrane of the host cell. However, the contents of the parasite’s apical organelles are secreted during invasion, and it is possible that some of this material is also incorporated into the nascent PVM (8). Since the RBC lacks the machinery to synthesize new lipids or proteins as the parasite grows over the next 48 h from 1 to 8 μm, the PVM is expanded and modified by the parasite, providing a customized environment for development (5). The PVM is closely associated with the parasite plasma membrane, so it is difficult to isolate the two membranes for direct proteomic and functional analysis, but a number of PVM-associated proteins have been identified by immunofluorescence assays (IFAs) and immunoelectron microscopy (IEM) (30, 32, 34). In intraerythrocytic asexual stages, EXP-1 (PF11_0224) and the ETRAMP family are the only well-characterized integral PVM proteins in *Plasmodium falciparum*, and Pf16 is the best-studied gametocyte-specific PVM protein (19, 29). EXP-2 and Pfmdv1/Pfpeg3 have also been reported to associate with the PVM, but neither have both a secretory signal sequence and an additional transmembrane domain (13, 19). Recently, PIETRAMP 10.3 (PF10_0164) has been shown to be essential for RBC stage growth, and in murine malaria, *Plasmodium berghei* UIS4, which is expressed on the PVM in liver stages, is also essential for growth (21, 26). *P. berghei* or *Plasmodium yoelii* sporozoites lacking UIS4 are able to invade liver cells and begin to grow, but die before producing new merozoites. Interestingly, the immune response induced against UIS4-deficient liver-stage parasites protects the mice against subsequent malaria infections and is being used as a model for the development of genetically attenuated vaccines (26). These findings have increased interest in the identification of additional liver-stage PVM proteins in *P. falciparum*; however, direct analysis of this stage is limited by the lack of a good in vitro culture system.

One approach to identify proteins targeted to a specific location is to determine the signaling motif that directs trafficking. This strategy is used to identify proteins targeted to the apicoplast, mitochondria, or secretory pathway as well as pro-

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‡ Supplemental material for this article may be found at http://ec.asm.org/.
* Published ahead of print on 15 April 2011.
teins exported across the PVM into the RBC (16, 22). The export motifs (PEXEL and HT) identified were found in a large number of Plasmodium genes (329 and 214, respectively) and revealed the extent to which the parasite modifies the host RBC (PlasmoDB 7.1). Similarly, determination of the signal required to selectively transport a protein from the parasite to the PVM should facilitate the identification of additional PVM proteins. To directly evaluate the regions required for PVM targeting and retention, the gametocyte-specific PVM protein Pfs16 was selected. It is small (16 kDa) and abundantly expressed through the 10 to 12 days required for P. falciparum gametocytes to mature from a stage I to stage V gametocyte (4, 20). Targeting can be monitored throughout gametocyte development and during gametogony, the transition from a mature, sausage-shaped, intraerythrocytic stage V gametocyte to a spherical extracellular gamete, which takes place in the mosquito. The molecular mechanisms involved in gametogenesis are still not well understood, but previous EM data have shown that gametocytes first round up, then the PVM is degraded, and finally the RBC membrane is released (28). The green fluorescent protein (GFP) reporter lines developed in this study reveal the amino acid sequences required for PVM targeting through gametocyte development and RBC emergence.

MATERIALS AND METHODS

*Plasmodium falciparum* parasites. Wild-type and transformed *P. falciparum* parasites (strain 3D7) were maintained in culture, and gametocyte-toxogony was induced as described by Ifediba and Vanderberg (17). Briefly, cultures were set up on day 0 at 0.1% parasitemia and 6% hematocrit. On day 3, the amount of medium was doubled, effectively decreasing the hematocrit to 3%. The gametocyte cultures were fed daily for the next 2 weeks, and gametocyte development was monitored by Giemsa-stained thin smears of the cultures. Mature stage V gametocytes develop by day 16 and can be stimulated to undergo gametogenesis by decreasing the temperature below 30°C and adding serum or xanthurenic acid (100 μM).

Reporter constructs. To produce the five Pfs16-GFP reporter constructs, the Pfs16 gene was amplified beginning 784 bp upstream from the ATG and extending to by 93, using primers Pfs16.-784.s and Pfs16.93.a (Table 1 and Fig. 1). For the construct that did not contain the carboxy (C)-terminus of Pfs16 (NC), the Pfs16.-784.93 PCR product was inserted between the SacII and XbaI sites of pDH.TgA.22.Pfs16-784.93 plasmid. Following insertion, the GFP coding region was expressed through the 10 to 12 days required for Pfs16-GFP reporter constructs production.

| Primer | Sequence |
|--------|----------|
| Pfs16.-784.s | 5'-TAATCCGCGCCGCAATAGTTATAGAGCA-3' |
| Pfs16.93.a | 5'-TAAATCTAGATGCTTGTGCAATGGCTGA-3' |
| GFP.1.s | 5'-TAATTTCAATGTCATGCTAAAGGAAG-3' |
| GFP.720.a | 5'-TAATGGATCTCCTGCGCCCTTTGTTAGTGTACTCCATGCC-3' |
| Pfs16.94.s | 5'-TAATGCGGCCAAGACGCCGCTGAAAAGGA-3' |
| Pfs16.480.a | 5'-TAATGGCGCCCTTAAAGAATGCATCTGGTGC-3' |
| Pfs16.312.s | 5'-TAATGGCGCCCTTAAATCTGTGCATCTGGTGC-3' |
| Pfs16.480B.a | 5'-TAATGGATCTCCTAAGATCTCCTTCATGTAATTATCTTTATCTTTATAC-3' |
| Pfs16.438.a | 5'-TAATGGCCGCTTAAATCTGTGCATCTGGTGC-3' |
| Pfs16.384RKAAA.a | 5'-TAATGGCGCCCTATATGCTGTCGTTTATCAATATCCAATATACCAGATAAT-3' |

The reporter lines were assayed directly by epifluorescence microscopy (Axiovert 200/Axiovision 4.3; Zeiss, Thornwood, NY) to evaluate GFP expression, and DNA was visualized by the addition of 1 μg ml^-1 bisbenzimide to the sample.

**A** Pfs16-GFP reporter constructs

| nums | FL | FC | TC | TM |
|------|----|----|----|----|
| 1) FL | -784 | -1 | GFP | Core | Tm |
| 2) FC | -784 | -1 | GFP | Core | Tm |
| 3) TC | -784 | -1 | GFP | Core | Tm |
| 4) TM | -784 | -1 | GFP | Core | Tm |
| 5) NC | -784 | -1 | GFP | Core | Tm |

**B** Pfs16 amino acid sequence

| nums | Core | Tm |
|------|-----|-----|
| 1) FL | 50 | MMC|E|PSL|KLML|F|FA|ANUL|SD|ANDK|GFP |
| 2) FC | 50 | KKM|CP|GSP|STL|QT|PGSSSGASLH|AV|PGNO|GLG|GQ |
| 3) TC | 50 | LSGK|DA|KMP|L|TY|GL|EM|KSL|SM|MD|K|TTV|NR|
| 4) TM | 50 | LIGS|TV|MIL|IL|SG|VV |
| 5) NC | 50 | ND | GE |GDO |US |

FIG. 1. Schematic of the Pfs16-GFP reporter constructs. The five Pfs16-GFP reporter constructs are depicted in panel A, and the amino acid sequences of the different sections of the Pfs16 coding region included in the constructs are depicted in panel B and color coded. All of the constructs contain 784 bp upstream from the ATG of the Pfs16 coding region (~ 784 to 1 [yellow]), the N-terminal (N-term) 93 bp encoding the first 31 aa that contain the predicted secretory signal sequence (Nt [purple]), and the GFP coding region (green). The full-length construct contains the region between the Nt domain and the transmembrane domain (core [light blue]), the transmembrane domain (Tm [dark blue]), and all four sections of the C-terminal (C-term) domain (sections 1 to 3 in red and section 4 in orange). In addition to the transmembrane region, the Tm construct contains an additional 5 C-terminal aa, RKAAC (white box), and the NC construct contains a stop codon at the end of GFP.
RESULTS

PVM-associated protein sequence analysis. PVM-associated, integral membrane proteins, EXP1, Pf16, and the ETRAMP family proteins are small proteins with a predicted secretory signal sequence, followed by a lysine-rich region and then a transmembrane domain and C-terminal tail enriched in charged amino acids, including lysine and aspartate. Comparison of the complete sequences of Pf16, EXP1, and a representative ETRAMP member, PF10_0164, using Glam2 software identified a conserved motif in the C-terminal region of the protein, beginning at the end of the predicted transmembrane domain (14) (Fig. 2A). Only a single ETRAMP protein was used in the search to avoid identifying motifs specific for ETRAMP family members not in the PVM. Twenty-one proteins were identified when this motif was used to screen the 953 proteins annotated in PlasmoDB to contain a secretory signal sequence and not an RBC export domain (see Table S1 in the supplemental material). The gene set included the original three genes and five other genes that have been shown to be associated with the PVM: three additional ETRAMP proteins (PFE1590w, PFD1120c, and PF10_0019); the translocon component PTEX150 (PF14_344), which is involved in exporting proteins associated with the apical organelles in the PVM; and five other ACS genes, PFL2570w, PFC0050c, and PFL0050c, which are members of the P. falciparum-specific acyl-CoA synthetase 9 (ACS9) family. Of these, PFL2570w (ACS3) has been localized to the PVM with PF14_0761 (ACS1), but the localization of the rest of the family has not been determined (35). To further evaluate whether this domain is involved in PVM trafficking, the C-terminal region of Pf16 containing the motif was directly tested for its ability to target PVM association.

Mapping Pfs16 trafficking signals. Five Pfs16-GFP reporter constructs were generated containing distinct regions of Pfs16 (Fig. 1A). The reference construct included the entire gene under the control of the homologous promoter (from −784 bp from the ATG to the stop codon at +471 bp) with the coding sequence of GFP inserted between bp 93 and 94 (full length [FL]). In the next construct (full C terminus [FC]), only the coding region for the C-terminal 53 aa was included to GFP. This region of the gene includes the predicted transmembrane domain (aa 105 to 126), and the remaining 31 C-terminal aa (aa 125 to 157) and contains the entire motif identified by Glam2. Further analysis of the amino acid sequence and charge composition of the C-terminal domain indicates that it can be divided into four sections with distinct charge profiles. Section 1 includes the first 7 aa following the transmembrane domain and includes 4 positively charged aa, 4 lysines (K); section 2 includes the next 7 aa and contains 3 tandem aspartates (D) followed by K-glycine-D; section 3 includes the next 6 aa, which is made up of three tandem repeats of KD; and section 4 includes the last 11 aa and includes three sets of 2 negatively charged aa (DD or D-glutamate [E]) separated by a glycine (Fig. 1B). The third construct (truncated C terminus [TC]) lacks section 4, the final 11 C-terminal aa, and increases the ratio of positive to negative amino acid. The fourth construct (transmembrane domain [TM]) contains only the transmembrane domain and 5 additional aa (RKAAA), and the fifth and final construct (no C-terminal amino acids [NC]) does not include any C-terminal amino acids after GFP.

P. falciparum strain 3D7 parasites were transformed with the five constructs, and the GFP expression profile was followed through gametogenesis. For all constructs, fluorescence was observed in a small subpopulation of schizonts (<1%) that are predicted to contain merozoites committed to sexual differentiation following RBC invasion. Expression continued as the parasites developed through the five stages of gametocytogenesis (I to V) (Fig. 3). The strongest fluorescent signal was around the periphery of the parasite for all constructs except Tm, which had such a weak signal it was hard to localize. To this predicted to be targeted to the apicoplast. Of the five predicted to be localized to the apicoplast, only the location of cpf60 in the apicoplast has been confirmed experimentally.

Motif identification can be complicated by the high frequency of regions of low amino acid complexity in P. falciparum; therefore, the motif domains identified in the eight known PVM proteins were reanalyzed with Glam2 software and the refined domain was used to search the same set of secreted, not exported, genes. Other than the PVM proteins used to define the motif only 2 of the 15 genes found in the first screen were identified, PF10_0242 and PFE0560c, indicating a closer association with the PVM proteins than the other genes initially identified. The refined motif also identified three additional ACS genes, PFL2570w, PFC0050c, and PFC0685c, which are members of the P. falciparum-specific acyl-CoA synthetase 9 (ACS9) expansion (3). One of these, PFL2570w (ACS3) has been localized to the PVM with PF14_0761 (ACS1), but the location of the rest of the family has not been determined (35). To further evaluate whether this domain is involved in PVM trafficking, the C-terminal region of Pf16 containing the motif was directly tested for the ability to target PVM association.
Gametocyte production was not altered in the Tm line, but there was little fluorescence, suggesting that the reporter protein was either not produced well or rapidly degraded, and consequently it was not included in further analysis. Another difference between the fluorescence patterns of the constructs was the presence of a prominent punctate dot in the gametocytes transformed with the GFP reporter construct without any C-terminal amino acids (NC). The identity of this structure is unknown, but it could be an organelle involved in trafficking secreted proteins to the PV.

Pfs16 membrane association. To test membrane association more directly, the solubilities of the Pfs16-GFP reporter construct...
Proteins were tested. Gametocytes (stages III to IV) from the different transformed lines were purified on a 16% Nycodenz cushion and lysed by 3 freeze-thaw cycles in hypotonic buffer. The soluble material was isolated by centrifugation, and the remaining insoluble pellet was extracted in 1% Triton X-100. Immunoblots of the soluble and Triton X-100-extracted material were then probed with anti-GFP antibodies (Fig. 4). In the absence of the C-terminal amino acids, the Pfs16-GFP reporter protein (NC; 31 kDa) was found in the soluble fraction, while the full-length Pfs16-GFP (FL; 44.1 kDa) and full-length C-terminal tail Pfs16-GFP (FC; 36.7 kDa) were found primarily in the Triton X-100-soluble fraction. The truncated C-terminal tail Pfs16-GFP reporter protein (TC; 35.6 kDa) was found in both fractions, suggesting the lack of the 11 C-terminal aa decreased PVM association.

Consistent with the differences in membrane retention demonstrated by immunoblotting, different fluorescent patterns were observed in the morphologically aberrant gametocytes that appear during routine culture and are assumed to be dying. In parasites transformed with either the full-length Pfs16-GFP (FL) or the full-length C-terminal tail (FC), a strong fluorescent signal was seen in various membranous structures within the infected RBC (Fig. 5). In parasites transformed with the reporter construct that lacked the C-terminal amino acids (NC), there was an increase in punctate fluorescent staining that appeared to be associated with the parasite. Both fluorescent patterns were seen in parasites transformed with the truncated C-terminal tail Pfs16-GFP reporter (TC), although in this case, the extra parasitic membranes were less fluorescent than those seen for the FL and FC constructs.

Tracking Pfs16 through gametogenesis. During gametogenesis, the final step in sexual differentiation, the mature gamete emerges from both the PVM and RBC membrane prior to fertilization. In the field, this only happens in the mosquito midgut, but it can be simulated in vitro by exposing mature stage V gametocytes to temperatures below 30°C in the presence of human serum (2). Under these conditions the sausage-shape gametocyte rounds up, the PVM degrades, and the RBC membrane ruptures, releasing the gamete for fertilization. Pfs16 is known not to be expressed on the surface of the emerged gamete, but the location of the protein has not been tracked as the gametocyte rounds up and emerges. Emergence is complete in 20 min after the stimulus, so to visualize the early events, wild-type parasites were fixed in 1% formalde-
hyde–PBS 0, 5, 15, and 20 min after the stimulus and then processed for IFA (Fig. 6A). As the wild-type gametocyte begins to round up, Pfs16 concentrates at the ends of the gametocyte and then continues to aggregate within the RBC as it is released (Fig. 6). To evaluate which region of Pfs16 was required for this pattern, the Pfs16-GFP reporter lines were tested. The pattern observed by IFA was mimicked by full-length Pfs16-GFP (Fig. 6A), but not the other constructs (Fig. 7). None of the other reporter constructs concentrated at the end of the rounding gametocyte, suggesting that this may require amino acids on the amino-terminal side of the transmembrane domain. The two other reporter constructs with the C-terminal tail (FC and TC) that associated with the PVM did aggregate during RBC release, while the reporter constructs without the C terminus (Tm and NC) did not. This is consistent with FC and TC Pfs16-GFP reporter protein remaining associated with the residual PVM membranes that are not completely removed until the RBC membrane is released.

**DISCUSSION**

The Pfs16-GFP reporter constructs demonstrate that the first 31 N-terminal aa of Pfs16 target secretion to the PV and that inclusion of the predicted transmembrane domain and C-terminal aa 127 to 146 is sufficient for association with the PVM. The further addition of the last 11 C-terminal aa increases retention on the membrane. This localizes the signal for PVM targeting to a 42-aa region made up of the 22-aa transmembrane domain and the first 20 aa of the 31 aa C-terminal tail (aa 127 to 146) and is the first functional identification of a peptide domain that targets a reporter protein to the PVM. The further addition of the acidic tail amino acids (aa 147 to 157) increases membrane association, suggesting that it is involved in an interaction that stabilizes the protein in the membrane.

The 53-aa region of Pfs16 required for optimal PVM retention includes the complete 42-aa motif that was identified using the Glam2 program to compare the complete amino acid sequences of three known PVM proteins, Pfs16, EXP1, and ETRAMP 10.3. The motif includes 12 aa from the transmembrane domain followed by 30 additional aa which are highly enriched in charged residues, 8 K, 11 D, and 2 E. The ability of the motif to identify five other genes, three additional ETRAMPs, PTEX 150, and acyl-CoA synthetase (PF14_0761) that have been shown to be associated with the PVM (see Table S1 in the supplemental material) lends support to the association of this domain with the PVM (Table S1). The general structure of the amino acid domain identified by the motif is a hydrophobic domain followed by a region enriched in charged amino acids. This basic architecture is shared by proteins targeted to the apicoplast and the TRAP protein family, the members of which are trafficked to the micronemes and then to the plasma membrane during invasion (24, 32, 37). The distinctive feature of the PVM-associated region identified here as well as the C-terminal region of PVM-associated proteins EXP-2 (PF14_0678), PMDV1 (PFL0795c), and the other ETRAMP family members is the presence of both basic and acidic residues, instead of the predominately basic amino acids associated with apicoplast targeting or the acidic C-ter-
minal regions of the TRAP family. The aldolase binding site found in the TRAP family is also not present in the PVM proteins. Such charged regions are often involved in protein-protein interactions, and it is possible that different parts of the domain contain different distinct trafficking signals. The C-terminal region of Pfs16 or the other PVM proteins were not included in the sequences tested in the yeast two-hybrid screen of *P. falciparum*, so additional experiments are needed to identify possible binding partners (18). The identification of the experimentally defined PVM targeting region of Pfs16 reported here allows further dissection of the trafficking pathway to be focused on this domain.

Two additional genes, PF10_0242 and PFE0560c, were identified both in the initial search and when the search was refined by using the motif found in the eight PVM proteins identified in the first search. Neither gene has been well studied to date,
and their location has not been determined experimentally. PFE0560c transcript is maximal in trophozoites, and it contains membrane organization and recognition nexus (MORN) repeats, which are often involved in regulating membrane-cytoskeleton interactions. Another *P. falciparum* MORN repeat protein, PF10_0306, has been shown to be associated with inner membrane complex formation during cell division, but only PFE0560c was identified in the PVM motif search, consistent with the proteins being targeted to different locations (12). There is proteomic evidence for PF10_0242 expression in gametocytes and asexual stages, and it is predicted to be an integral membrane protein, but it does not have functional similarity to other proteins. Importantly, there is no evidence that PF10_0242 or PFE0560c is expressed in salivary gland sporozoites or liver stages, so it is unlikely that they are functional orthologs of *P. berghii* UIS4, a candidate for a genetically attenuated malaria vaccine. Only two identified genes were maximally expressed in salivary gland sporozoites: one gene in the first search, coding for TRAP-like protein (TLP [PF0800w]), and one gene in the second search, coding for MSP7-like protein (PF13_0191). However, the second search also identified the oocyst sporozoite gene (PFF0620c), which contains a 6-Cys domain and is predicted to have a glycosylphosphatidylinositol tail. These genes are not highly homologous to the *P. falciparum* ortholog of UIS4, but if in vitro culture systems for *P. falciparum* liver stages improve, it would be interesting to evaluate their location directly. ETRAMP 10.3 was suggested to be the ortholog of UIS4 based on chromosomal synteny, but a recent study found ETRAMP 10.3 was essential for asexual development in *P. falciparum*. The critical role of the PVM has been demonstrated in both the liver stages and intraerythrocytic parasites (21, 27). From its beginning as an invagination of the host plasma membrane, the PVM expands and is modified as the intracellular parasite continues the life cycle by invading another cell or, in the case of a gametocyte, fertilization and the beginning sporogonic development. This work demonstrates that the 53 C-terminal aa of Pfs16 target PVM expression and retention during gametocyte development. This would be consistent with the N-terminal region of Pfs16 being located in the PV and possibly interacting with the gametocyte surface so that it moves as the gametocyte changes shape. The C-terminal region of Pfs16 is likely to be on the RBC side of the PVM and is required to retain the protein in the membrane, possibly due to a charge effect or by interacting with a protein on the RBC face of the PVM.

The close contact between the parasite plasma membrane and the PVM is clearly demonstrated by the similarity of the fluorescent pattern of the soluble secreted form of Pfs16-GFP (NC) and full-length Pfs16 (FL) (Fig. 3). This intimate association suggests extensive interactions between proteins on the PPM and PVM and dual regulation of growth. Interestingly, one of the first signs of a dying gametocyte is the appearance of additional extraparasitic membranes (15). The presence of GFP-labeled Pfs16 suggests that these are derived at least in part from the PVM, although the presence of parasite plasma membrane cannot be excluded. The structures formed are reminiscent of the membrane blebbing seen in apoptosis in mammalian cells (39). Possibly the parasite employs a similar strategy of maintaining membrane integrity until the intracellular contents are completely degraded to avoid releasing intact proteins and stimulating an immune response. As a result, the shrinkage of the parasite without a corresponding decrease in the amount of membrane gives rise to the altered morphology so clearly observed in the Pfs16-GFP tagged cells.

The critical role of the PVM has been demonstrated in both the live stages and intraerythrocytic parasites (21, 27). From its beginning as an invagination of the host plasma membrane, the PVM expands and is modified as the intracellular parasite grows. After maturation, both the PVM and host cell plasma membrane must rupture for the parasite to emerge and continue the life cycle by invading another cell or, in the case of a gametocyte, fertilization and the beginning sporogonic development. This work demonstrates that the 53 C-terminal aa of Pfs16 target PVM expression and retention during gametocytogenesis and that all known PVM proteins were found to have C-terminal regions with a similar charge structure. Similarities in the amino acid sequence of this domain in proteins expressed at different parasite stages suggest a similar PVM trafficking pathway is used throughout the life cycle. To focus further functional evaluation and identify new potential PVM proteins, a 34-aa motif was identified in the C-terminal region of eight PVM proteins, including Pfs16. Removal of the 10 C-terminal aa of this motif from the Pfs16-GFP reporter protein did not affect trafficking but reduced retention on the PVM membrane, suggesting that this region is involved in stabilizing the protein-membrane interaction. In contrast, the 53-aa PVM targeting domain was not sufficient for Pfs16-GFP capping to the ends of the gametocyte, indicating another region of the protein is required. The fine structure of the PVM targeting signal and the interacting proteins required for trafficking and membrane association can now be further evaluated to define the pathways used by the parasite to survive and thrive in the host cell.
ACKNOWLEDGMENTS

This investigation received financial support from Public Health Service grants AI069314 and AI48826 from the National Institute of Allergy and Infectious Disease and by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

We thank Beata Czesny and Amreena Suri for technical assistance and Margery Sullivan for editorial assistance.

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