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A 95 kDa protein of *Plasmodium vivax* and *P. cynomolgi* visualized by 3-D tomography in the caveola-vesicle complexes (Schüffner’s dots) of infected erythrocytes is a member of the PHIST family

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Summary

*Plasmodium vivax* and *P. cynomolgi* produce numerous caveolae-vesicle complex (CVC) structures within the surface of the infected erythrocyte membrane. These contrast with the electron-dense knob protrusions expressed at the surface of *P. falciparum* infected erythrocytes. Here we investigate the 3-dimensional structure of the CVCs and the identity of a predominantly expressed 95 kDa CVC protein. Liquid chromatography - tandem mass spectrometry analysis of immunoprecipitates by monoclonal antibodies from *P. cynomolgi* extracts identified this protein as a member of the *Plasmodium* helical interspersed sub-telomeric (PHIST) superfamily with a calculated mass of 81 kDa. We named the orthologous proteins PvPHIST/CVC-8195 and PcyPHIST/CVC-8195, analyzed their structural features, including a PEXEL motif, repeated sequences and a C-terminal PHIST domain, and show that PHIST/CVC-8195 is most highly expressed in trophozoites. We generated images of CVCs in 3-D using electron tomography, and used immuno-Electron Tomography (ET) to show PHIST/CVC-8195 localizes to the cytoplasmic side of the CVC tubular extensions. Targeted gene disruptions were attempted in vivo. The pcyphist/cvc-8195 gene was not disrupted, but parasites containing episomes with the tgdhfr selection cassette were retrieved by selection with pyrimethamine. This suggests that PHIST/CVC-8195 is essential for survival of these malaria parasites.
Keywords
Malaria; Plasmodium; Erythrocytes; Tomography; PHIST; Caveolae-Vesicle Complex

Introduction
Malaria, an infectious disease caused predominantly by Plasmodium vivax and P. falciparum, results in illness in more than two hundred million people each year in over one hundred countries (WHO, 2010). The parasite undergoes a cyclical process of invading, thriving within, and rupturing host erythrocytes. During the blood-stage of the disease, the merozoite form of the parasite efficiently enters host red blood cells (RBCs), grows and multiplies to create new asexual progeny, and also differentiates to generate sexual stage parasites. Pathogenic processes ensue in the host, with resulting organ damage, anemia and associated medical complications (reviewed in Price et al., 2009; Mueller et al., 2009; Rowe et al., 2009). The biological mechanisms used by P. vivax and P. falciparum to invade, grow and survive inside RBCs have important species-specific differences, which are largely unexplored. Research on P. vivax has trailed far behind P. falciparum, and comparative studies have been few.

As a merozoite is invading a target host RBC it creates a parasitophorous vacuole (PV) (reviewed in Galinski et al., 2005). The parasite grows within the PV, surrounded by a PV membrane, the host RBC cytoplasm and host RBC membrane. Although the uninfected, enucleated RBC lacks protein synthesis and trafficking machinery, the parasite has the capacity to produce and transport proteins to the PV membrane and onwards using specialized structures it develops in the RBC cytoplasm and at the surface of the iRBC membrane. Investigations in this area of malaria biology are most advanced for P. falciparum, resulting in many breakthroughs in the scientific understanding of transport biology in this species (reviewed in Tilley et al., 2008; Maier et al., 2009; Haase et al., 2010; Tilley et al., 2011). Research on P. falciparum blood-stage biology has been aided since 1976 by the availability of robust in vitro culture systems (Trager et al., 1976), and, over the last decade by a P. falciparum genome database (Gardner et al., 2002) and a variety of functional genome technologies (reviewed in Di Girolamo et al., 2005; Aurrecoechea et al., 2009). A growing body of knowledge now exists relating to the structure and composition of P. falciparum-induced cytoplasmic membranous structures that develop between the PV and the surface of the iRBC. Notably, the P. falciparum iRBC membrane surface also becomes studded with electron-dense structures known as knobs, which contain erythrocyte membrane protein-1 (PfEMP-1), the antigenically variant virulence protein that is encoded by the large var multigene family (reviewed in Scherf et al., 2008). Recently, electron tomography (ET) has been employed for imaging P. falciparum iRBCs, and this technology has enabled 3-dimensional (3-D) imaging of the whole iRBC, Maurer’s cleft organelles and the discovery of tethers bridging them to the iRBC membrane (Hanssen et al., 2008; Hanssen et al., 2011).

In contrast to developing protruding knob structures, peppered along the surface of the iRBC host membrane, P. vivax induces a pitted membrane surface with the formation of numerous flask-shaped indentations of the iRBC membrane, called caveolae, in association with vesicles. These unique intricate structures, termed caveola-vesicle complexes (CVCs), were first observed by transmission electron microscopy (TEM) in 1975 (Aikawa et al., 1975) and described as 90–100 nm wide caveolae with several 40–50 nm wide electron-dense vesicles extending from the caveolar base. Some vesicles appear alveolar in shape, while others are elongated and tubular (Aikawa et al., 1975). These studies, which focused on the iRBC membranes of P. vivax and the simian malaria parasite P. cynomolgi, a species that is
genetically very close to *P. vivax* (Waters *et al.*, 1993), also established that the caveolae structures are open to the exterior of the host cell and physically accumulate Giemsa stain resulting in the abundant reddish speckled appearance in *P. vivax* iRBCs treated with Romanowsky-based stains known as Schüffner’s stippling (Schüffner, 1899). CVCs have since also been confirmed by TEM to be present in the human malaria species *P. ovale* (Matsumoto *et al.*, 1986; Aikawa *et al.*, 1977), and, based on similar Giemsa-stained patterns, they are presumed to be present in at least three other non-human primate species of *Plasmodium* (*P. fieldi*, *P. simiovale* and *P. gonderi*); the iRBCs of each of these species are similarly characterized by profuse Schüffner’s like stippling of Giemsa-stained iRBCs in blood smears (Coatney, 1971).

How CVC form and the functional purpose of these abundant membrane structures are not known, although it has been suggested that they may function in nutrient transport or release of parasite metabolites from infected erythrocytes (Aikawa *et al.*, 1975; Udagama *et al.*, 1988; Matsumoto *et al.*, 1988). Limited exploratory biochemical studies confirmed that they can provide a means for metabolic exchange of the parasitized cell with the host plasma (Aikawa *et al.*, 1975). Lactoperoxidase catalyzed $^{125}$I surface examination, metabolic radiolabeling experiments and immunobiochemical studies verified that the CVCs are in part comprised of a 95 kDa and other specific parasite-derived proteins (Barnwell *et al.*, 1990; Udagama *et al.*, 1988; Barnwell, 1986). However, the composition, developmental origin, and function(s) of the CVCs remain otherwise largely unexplored. The simian malaria species *P. knowlesi*, which is also related to *P. vivax*, in contrast, only produces a few caveolae at the surface of the iRBCs, without associated alveolar or tubular vesicles (Aikawa *et al.*, 1975).

Investigations relating to the unique species-specific modifications of *P. vivax* iRBCs and corresponding transport biology have not progressed in parallel with similar lines of research on *P. falciparum* iRBCs. This is largely because continuous in vitro blood-stage culture systems and routine functional genetic technologies have not been established for studies of *P. vivax* or for *P. cynomolgi*, which serves as an effective and reliable experimental model for *P. vivax* (reviewed in Galinski *et al.*, 2008; Galinski *et al.*, In press). Moreover, the first *P. vivax* genome sequence and associated database was published only recently (Carlton *et al.*, 2008), and a *P. cynomolgi* genome sequence has not yet been published. While functional genomics for these species is in its experimental infancy, initial successful transient transfections of *P. vivax* (Pfahler *et al.*, 2006) and *P. cynomolgi* (Kocken *et al.*, 1999) iRBCs have been reported in experimental infections of non-human primates.

In the present study, we aimed to investigate the 95 kDa protein that was shown to be a predominant parasite-derived component exclusively associated with the CVCs in *P. vivax* iRBCs (Barnwell *et al.*, 1990), and also examine the structure of the CVCs for the first time in 3-D. Liquid chromatography - tandem mass spectrometry (LC-MS/MS) analysis of this protein immunoprecipitated from detergent extracts of *P. cynomolgi* iRBC using monoclonal antibodies (mAbs) revealed it to be a member of the *Plasmodium* Helical Interspersed Sub-Telomeric (PHIST) superfamily of proteins (Sargeant *et al.*, 2006) with a calculated mass of 81 kDa. Electron tomograms are presented showing the elaborate multi-vesicular/tubular nature of the parasite’s CVCs and confirming the presence of the 95 kDa protein, now termed PHIST/CVC-81$_{95}$, specifically on the cytoplasmic side of the tubules. Finally, we demonstrate that the PHIST/CVC-81$_{95}$ protein appears to be required for growth of the parasite. Transfected *P. cynomolgi* parasites could be retrieved from in vivo rhesus monkey blood-stage infections after applying pyrimethamine drug pressure, with eposomes containing the pcyphist/cvc-81$_{95}$ knock out (KO) construct and the tgdhfr drug selection cassette, but we were not able to achieve disruption of the pcyphist/cvc-81$_{95}$ gene.
### Results

**Proteomic identification of the predominant *P. vivax* 95 kDa CVC protein as a member of the PHIST superfamily by detection and analysis of its homolog in *P. cynomolgi* iRBCs**

A subset of mAbs developed against mature *P. vivax* iRBCs were shown previously to target specifically the CVCs in *P. vivax* iRBC membranes and to immunoprecipitate from SDS extracts of iRBCs a predominant *P. vivax* antigen that migrated at 95 kDa in SDS-PAGE (Barnwell et al., 1990; Barnwell, 1986). Several of these mAbs were also reported at the time to cross-react in indirect immunofluorescence assays (IFA) with *P. cynomolgi* iRBC. *P. cynomolgi* iRBCs are easily generated in large quantities from rhesus monkey infections and therefore more amenable to in depth study than *P. vivax* attained from small New World monkey infections or clinical isolates. We set out in the current studies to use four of the mAb reagents in proteomic experiments to identify the associated gene in *P. cynomolgi* and further investigate the structure, location and function of this predominant protein in the context of the CVCs. To proceed, first we reconfirmed the crossreactivity of mAbs 2H12.B4, 2H8.E10, 4C12.G4, and 1H4.B6 with *P. cynomolgi* trophozoite iRBCs (Fig. 1A). The typical fluorescence pattern representative of CVCs with a dense pattern of speckling was reliably produced. These heavily dotted patterns mimic the classic spread of pink to dark red dots of Schüffner’s stippling observed throughout *P. vivax* and *P. cynomolgi* iRBCs in Giemsa-stained thin blood smears.

We confirmed that all four mAbs (2H12.B4, 2H8.E10, 4C12.G4, and 1H4.B6) recognized the expected 95 kDa antigen, in SDS extracts of *P. cynomolgi* iRBCs by immunoblot analysis (Fig. 1B), and immunoprecipitated the corresponding protein and associated breakdown products. The *P. cynomolgi* 95 kDa protein was immunoprecipitated with each mAb, excised and processed from the SDS-PAGE gel slices and analyzed by LC-MS/MS. The resulting peptide sequences were searched against the *P. vivax* genome database and the gene ID: PVX_093680 (annotated as a member of the PHIST superfamily; Sargeant et al., 2006) was identified with each antibody reagent. This PHIST protein, like all known members of the superfamily, is characterized by the presence of a PHIST domain, containing multiple predicted alpha helical domains and several conserved tryptophan residues. A representative immunoprecipitation result using mAb 1H4.B6 is shown in Fig. 1C, with the detection of the 95 kDa PHIST protein and a breakdown product at 37 kDa; the other major protein bands were verified by LC-MS/MS to be IgG. The MASCOT tool was used to search the *P. vivax* proteome database and the gene ID: PVX_093680 was identified with at least 22 spectral counts, 10 unique and no shared peptides. PVX_093680 has 2,133 nucleotides (nt) of coding sequence and is contained within two exons. The 710 amino acid protein has a calculated mass of 80.73 kDa, lower than the 95 kDa extrapolated from its relative electrophoretic mobility in SDS-PAGE gels. Such differences are not unusual for *Plasmodium* antigens, which depending on the amino acid composition and imposed secondary structure can (and more often than not) migrate quite different from their calculated molecular masses.

To maintain the original designation of this protein as a 95 kDa antigen determined by SDS-PAGE (Barnwell et al., 1990), and recognize the calculated molecular mass of 81 kDa, we hereafter refer to this protein in *P. vivax* and *P. cynomolgi* respectively as PvPHIST/CVC-81\textsuperscript{95} and PcyPHIST/CVC-81\textsuperscript{95}.

**PvPHIST/CVC-81\textsuperscript{95} and PcyPHIST/CVC-81\textsuperscript{95} lack species-specific repeated motifs present in other species while conservation of the PHIST domain is retained across species**

Fifty-two amino acids downstream of the N-terminus, PvPHIST/CVC-81\textsuperscript{95} has a 22 amino acid hydrophobic region. According to bioinformatic analyses using the MalSig algorithm...
This region may serve as a recessed signal sequence or signal anchor. A predicted *Plasmodium* export element (PEXEL; RxLxEx/Q/D; Marti *et al.*, 2004; Hiller *et al.*, 2004) follows approximately 12 amino acids downstream of this hydrophobic domain; this sequence has been shown to be important for the trafficking of some but not necessarily all proteins from the PV onwards to the surface of the iRBC (Spielmann *et al.*, 2010). The protein’s PHIST domain is at the C-terminal portion of the protein, with four predicted alpha helical domains and four positionally conserved tryptophan residues (Sargeant *et al.*, 2006). *PvPHIST/CVC-81* can also be classified as aspartate (11.7%) and glycine (11%) rich (http://ca.expasy.org/tools/protparam.html) due to the presence of remnants of degenerated repeated nucleotide sequence motifs within the central region of the gene (discussed further below) that encode a comparatively high number of these amino acids. Arginine and proline residues are also relatively high at 11.4% and 11%, respectively.

To support continued in depth investigations of *PvPHIST/CVC-81* and the biological and functional analysis of CVCs, we proceeded to identify and characterize the orthologous gene in the experimental model *P. cynomolgi*. The absence of a *P. cynomolgi* genome database at the time of this work necessitated a traditional scheme to identify and characterize the *pcyphist/cvc-81* gene using polymerase chain reaction (PCR) and standard DNA sequencing methods. First, *pvphist/cvc-81* homologs were detected in *P. falciparum* and the simian malaria species *P. knowlesi* through BLAST (Basic Linear Alignment Search Tool) nucleotide searches of the PlasmoDB database (http://plasmodb.org/plasmo/). The most closely related sequences identified have the respective gene IDs: Pf08_0137 (annotated as *Plasmodium* exported protein (PHISTc)) and PkH_011720, with calculated molecular masses of 147 kDa and 105 kDa, respectively; thus referred to here with the comparable PIPHIST-147 and PKPHIST-105 nomenclature. Both were annotated in the PlasmoDB database as proteins of unknown function. BLAST analyses of the NCBI database (http://www.ncbi.nlm.nih.gov/protein/) were also performed using the PvPHIST/CVC-81 amino acid sequence to reveal distant homologs from rodent malaria parasite species. *P. yoelii* (gene ID: Py01786) and *P. berghei* (duplicated gene IDs: PB108348.00.0 and PB000848.03.0 (Sargeant *et al.*, 2006)) homologs were identified; unlike the primate malaria parasite species, a family of paralogous genes is not present in these rodent parasite species.

Degenerate gene-specific and 3′UTR primer pairs were subsequently designed based on *pvphist/cvc-81* coding and non-coding regions (see experimental procedures) and the most closely related corresponding gene sequence from *P. knowlesi* (Pkh_011720; Phist-105). Subsequently, PCR amplification and cDNA sequencing experiments were performed to successfully identify the ortholog from *P. cynomolgi*. Our analyses verified the pcyphist/cvc-81 gene to be 2,433 nucleotides, encoding a 720 amino acid protein on two exons, with a similar basic structure as exists for the *P. vivax*, *P. falciparum* and *P. knowlesi* homologs (Fig. 2A). Notably, each has a hydrophobic sequence at the end of the first exon, a putative PEXEL motif (Marti *et al.*, 2004) at the beginning of exon II, a central region predominated by a variety of degenerated repeated sequences, and a conserved C-terminal PHIST domain with four characteristic alpha-helical domains and four tryptophan residues (Fig. 2A).

Our analysis shows that each of these proteins can be classified as members of the PHISTc subfamily, based on the positioning of the four conserved tryptophans within the PHIST domain (Sargeant *et al.*, 2006). In the *P. cynomolgi* gene, a 270 nucleotide intron separates the short initial exon of 213 nucleotides from the second exon of 1,950 nucleotides. The size of the exons and introns differs for each species, yet conserved sequence motifs and features define the homologs (Fig. 2B; the *P. knowlesi* phist-81 intron-exon boundaries are based

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on RNA sequencing information, Rayner and Galinski unpublished data). Table 1 shows the percentage of identities found at the nucleotide and amino acid levels when the entire P. vivax and P. cynomolgi PHIST/CVC-8195 sequences were aligned, compared to the three separate regions comprising these molecules at the DNA (5′ coding, central, and 3′ coding regions) and protein (N-terminal, central and C-terminal regions) levels. The N-terminal 155 amino acid regions (92%), and especially the C-terminal 155 amino acid regions (100%) encoding the PHIST domain, are highly conserved compared to the central region of the protein. Clustal alignments highlighting the relationship of the N-terminal and C-terminal regions of PvPHIST/CVC-8195, PcyPHIST/CVC-8195, PkPHIST-105 and PfPHIST-147 proteins are shown in Fig. 2B and Fig. 2C. Supplementary Information includes the sequences and alignments of these proteins, including the P. berghei and P. yoelii rodent parasite orthologs (Fig. S1 and Fig. S2). When the C-terminal PHIST domains are compared across all species at least three of four alpha helical domains (helix −1, −2 and −3) align, and the tryptophan residues are conserved in number in all species, including the rodent parasites (Fig. S1). The degenerate repeated motifs are readily apparent in the PfPHIST-147 and PkPHIST-105 proteins, and the rodent parasite PHIST proteins, but not in PvPHIST/CVC-8195 and PcyPHIST/CVC-8195. Only a few small 4-amino acid motifs (HDGX) are present in the P. vivax sequence and as well a number of doublets (e.g. DD, PP and GG) are repeated a few times in the central regions of both PvPHIST/CVC-8195 and PcyPHIST/CVC-8195. This suggests that the central regions of these genes are undergoing evolutionary changes that are not constrained by the requirement for strict conservation of the central domain. To the contrary, we hypothesize that the central regions of the PvPHIST/CVC-8195 and PcyPHIST/CVC-8195 proteins may have evolved to accommodate specific roles of these proteins in P. vivax and P. cynomolgi CVCs.

PcyPHIST/CVC-8195 is expressed throughout the erythrocytic stages in P. cynomolgi, predominantly in trophozoites

Once the pvphist/cvc-8195 and pcyphist/cvc-8195 genes and predicted proteins were identified and analyzed, a recombinant protein representing all 710 amino acids encoded by pvphist/cvc-8195 (rPvPHIST/CVC-8195) was expressed in Escherichia coli and used to produce a polyclonal rabbit antiserum (anti-rPvPHIST/CVC-8195). This antiserum was evaluated in immunofluorescence microscopy on thin blood smears containing P. cynomolgi iRBCs in different stages of its ~48 hour cycle. An immunofluorescence pattern of small dots spotted across the iRBC was evident beginning with moderate density at the ring-stage of development. The speckling became greatly increased in abundance and intensity in the trophozoite and schizont stages (Fig. 3A). Rabbit pre-immune serum was negative for all stages (data not shown). PcyPHIST/CVC-8195 was also detected by western immunoblot assays in each of these developmental stages with maximal expression apparent at the mature trophozoite stage (Fig. 3B). Interestingly, at the ring stage of development when the fluorescent labeled dots are sparser in number, an imperfect hexagonal plate or so-called “soccer ball” pattern of fluorescence was frequently apparent, with fluorescent areas intermixed with dark areas lacking fluorescence (Fig. 3A, panel 1, arrows).

Immunoelectron tomography reveals elaborate 3-dimensional signatures of the CVCs from P. cynomolgi-iRBCs and localizes PcyPHIST/CVC-8195 to the cytoplasmic side of the tubular extensions

Transmission EM of CVCs in cross-sectional analysis of P. vivax and P. cynomolgi iRBCs provide an incomplete picture of the physical nature of these organelles (Aikawa et al., 1975; Udagama et al., 1988, Matsumoto et al., 1988, Barnwell et al., 1990). Particularly with the definition of specific CVC proteins and antisera in hand, we became interested in exploring the CVC structures visually in 3-D space and determining the localization of the predominant PcyPHIST/CVC-8195 protein using immuno-ET methods. Electron
tomography enables the generation of 3-D images and we have previously shown this to be a powerful technique for visualizing internal membranous structure and organelles in *P. falciparum* iRBCs (Hanssen et al., 2008; Hanssen et al., 2010). Thus, *P. cynomolgi* trophozoite iRBC samples were processed in a similar manner, using equinatoxin II (EqII) (Anderluh et al., 1996; Jackson et al., 2004) treatment to release hemoglobin and allow entry of antibodies. Transmission electron micrographs and 3-D electron tomograms from intact and EqII-permeabilised cells were generated (Fig. 4, 5). The images revealed an elaborate structural nature of the CVCs. A caveolar “cup” with an electron-dense coat remains open to the surface of the iRBC and provides an open connection to a variable number of coated vesicles and tubules. The average width of the tubular structures is 40 nm, while the vesicles range in average diameter from 25 to 45 nm. Additional vesicles with similar diameters appear to be located close to the CVCs. The structures are similar in intact and EqII-permeabilised iRBCs indicating that this procedure does not compromise the CVC membrane structure, while permitting improved contrast. Other membranous structures are observed including Maurer’s cleft-like structures (Fig. 4C) and host cell mitochondria (Fig. 4A) consistent with the preference of *P. cynomolgi*, like *P. vivax*, for invading reticulocytes (reviewed in Galinski et al., 2008; Galinski et al., In press).

We demonstrate that PcyPHIST-81<sub>g5</sub> is associated with the CVCs of *P. cynomolgi* trophozoite iRBCs. Equinatoxin II-permeabilised iRBCs were labeled with mAb 4C12.G4 (Fig. 4D, 6 A–D) and anti-rPvPHIST/CVC-81<sub>g5</sub> serum (Fig. 4E, 6 E–H). The 4C12.G4 gold-labeled mAb localized to CVCs, as previously observed by immuno-TEM experiments with *P. vivax*-iRBCs (Barnwell et al., 1990). The labeling indicates that the epitope is present on the cytoplasmic side of the tubular extensions of the CVCs (Fig. 6D and 6H).

We have used IMOD to stitch serial 3-D reconstructed tomograms of a region of a *P. cynomolgi*-iRBC containing CVCs. The interconnected nature of the caveolar opening of the CVC, the body of the caveola and the connected multiple tubular extensions can be appreciated by examining individual sections from the electron tomograms (Fig. 5, 6) or by translating through the sections (Supplementary Videos S1, S3). Each CVC has a unique ‘morphological signature’, differing in the number and sizes of vesicles and tubules. IMOD was used to render the surface of the CVCs and 3-D rotations are presented in Supplementary Videos S2 and S4. The caveolar opening is rendered in blue and the tubule-vesicular extensions in orange.

**Genetic manipulation of *P. cynomolgi* with a pcyΔphist/cvc81<sub>g5</sub> vector**

Given the predominance of the PvPHIST/CVC-81<sub>g5</sub> and PcyPHIST/CVC-81<sub>g5</sub> proteins in the iRBC membrane and as a component of the CVCs (Barnwell et al., 1990), we hypothesized that this protein would prove to be essential for the parasite’s survival. To test this hypothesis, we carried out two transfection experiments aiming to target and knock out (KO) the pcyphist/cvc-81<sub>g5</sub> gene. The pcyΔphist/cvc-81<sub>g5</sub> KO cassette was designed to target 600 bp at the 5′-end and 554 bp at the 3′ end of the pcyphist-81<sub>g5</sub> gene (Fig. 7A). This construct also contains the mutated *tdgfr-ts* gene flanked by the *P. berghei* dhfr 5′ promoter and 3′ UTR regions to drive expression of the DHFR protein and confer pyrimethamine resistance. The pcyΔphist/cvc-81<sub>g5</sub> vector was digested with *BanHI* and *EcoRI* to release the KO cassette from its pUC19 backbone vector. The released, linearized constructs were visualized by agarose gel electrophoresis (Fig 7B). Each electroporation was performed using a suspension of *P. cynomolgi* schizont-iRBCs enriched from blood collected from an infected donor rhesus monkey. Transfected iRBCs were immediately reconstituted in incomplete RPMI-1640 and inoculated into a recipient rhesus monkey.

Parasitemia in recipient monkeys from two sequential experiments were monitored daily by microscopic analysis of thick and thin blood smears. For the first experiment, the

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parasitemia increased to 0.63% on day 3 and the first dose of pyrimethamine was administered. The drug was administered again on days 4 and 10. Parasites were not subsequently observed until day 14. Additional pyrimethamine doses were administered on days 17 and 18, to continue to select for parasites harboring the mutated tgdhfr-ts gene. The parasitemia increased to 3.1% by day 24, and the infected blood was collected, and stored. For the second experiment the parasitemia increased to 1.2% on day 6 and the first dose of pyrimethamine was administered. The drug was administered again on days 7, 8, 13, 14, 19, 20, 24 and 25. Selected parasites were observed by day 18. The parasitemia increased to 2.6% by day 25, and the infected blood was collected, and stored on day 26. Graphs of these parasitemias are shown in Fig. S3.

Genomic DNA of the recovered parasites was extracted and used as a template for PCR amplification to assess the integration status of the pcyΔphist/cvc-81_95 cassette, using pcyphist/cvc-81_95 forward and reverse primers (Fig. 7B). Despite the retrieval of pyrimethamine resistant parasites, these PCR tests were negative, indicating the lack of genomic integration of the pcyΔphist/cvc-81_95 cassette. Southern blots experiments were also consistent with the lack of integration (not shown). We then questioned whether episomes, possibly resulting from a low copy number of undigested plasmids in the electroporation mixture, were conferring resistance to the drug. To address this question, DNA from the transfected parasites was extracted using a QIAgen miniprep kit for plasmid purification. Plasmid DNA was retrieved from the iRBCs and used to transform TOP10 E. coli cells (invitrogen). Plasmid DNA was purified and digested with EcoRI and EcoRV. The digested products that were recovered (5.1 kb and 3.6 kb) corresponded to the pUC19/pcyΔphist81_95 vector used to transfect the P. cynomolgi iRBCs (Fig. 7C). As expected, IFA and western immunoblot experiments showed expression of the PHIST protein (Fig. 7D and Fig 7E). An experiment performed in parallel, on the other hand, served as a positive control, showing the integration and expression of a red fluorescent protein (rfp) gene (Fig. S4, and unpublished data). These data serve to confirm for the first time that integration of a transgene is possible in P. cynomolgi.

**Discussion**

Advances in post-genomic tools and microscopic methods have greatly expanded the potential of investigations on the cellular biology of Plasmodium iRBCs, particularly in P. falciparum. The recent publication of the P. vivax and P. knowlesi genome databases (Carlton et al., 2008; Pain et al., 2008), and focused efforts on sequencing P. cynomolgi genomes (unpublished), now raise the prospects for comparable discoveries in these species. This is especially important in light of increasing data showing that P. vivax, in addition to causing enormous morbidity worldwide, can cause severe disease and infections that may become lethal (reviewed in Mueller et al., 2009; Price et al., 2009).

*P. cynomolgi* iRBCs are the main focus of the exploratory studies reported here, because unlike *P. vivax* they are accessible from monkey (rhesus) infections in significant numbers for experimental manipulations and in-depth investigations. Based on knowledge regarding the close phylogenetic relationship of *P. cynomolgi* and *P. vivax* and their common overarching morphological and biological features, the protein composition and functional biology of specific organelles such as the CVC are expected to be extremely similar. The current experiments are a prelude to using *P. cynomolgi* as a surrogate in expanded cell biological studies relating to CVC ultrastructure, molecular composition, and function, and, to then advance specific questions directly with *P. vivax* iRBCs attained in limited quantities from small New World monkey infections.
This study is one of only a few since 1975 to investigate the structure and molecular make-up of Plasmodium CVCs (Aikawa et al., 1975; Barnwell et al., 1990; Barnwell, 1986; Aikawa et al., 1977; Atkinson et al., 1990; Bracho et al., 2006; Udagama et al., 1988; Matsumoto et al., 1988; Matsumoto et al., 1986) and the first to present these structures in 3-D. The ET imaging results presented here corroborate previous TEM reports showing numerous CVCs interspersed along the surface of the P. vivax and P. cynomolgi iRBCs with their caveolae open to the surface (Aikawa et al., 1975). The iRBC plasmalemma becomes highly reorganized over the course of the approximate 48-hour life span of the growing parasites to accommodate these intricate structures. Many interesting questions remain to be answered including 1) what proteins make up these structures, 2) how are they formed, 3) for what purpose(s), and 4) can they become targets of intervention?

Proteomic experiments were critical to identify the phist/cvc81\textsubscript{95} gene encoding the 95 kDa CVC antigen and designate this as a member of the PHISTc sub-family. While this protein was shown previously to be a predominant protein that is localized to the CVCs of P. vivax and P. cynomolgi, extractable primarily in SDS, and predicted to be associated with the cytoskeleton underlying the RBC membrane (Barnwell et al., 1990), its gene and encoded protein characteristics had remained unknown. This discovery in fact marks the first designation of a physical location of any member of the PHIST superfamily (Sargeant et al., 2006).

The tomograms show that the CVC opening (narrowing to a diameter of ~35 nm) has a thickened limiting membrane at the region of connection to the iRBC membrane; i.e. the electron dense caveola cup. There are 5–7 tubules (~40 nm in diameter) emanating from each CVC opening; these tubules vary in length and can be considerably elongated (up to 400 nm). Vesicles ranging in average diameter from 25 to 45 nm are also an integral part of the CVCs, while others appear in isolation nearby. Large Maurer’s cleft-like structures are also observed in the host cell cytoplasm. The cytoplasmic face of the tubules was clearly labeled with anti-PHIST/CVC-81\textsubscript{95} mAb and polyclonal antiserum in immuno-ET experiments, while the CVC openings, the CVC-associated vesicles and the cleft-like structures were not labeled. In previous immuno-TEM analyses, using post embedding immunolabeling procedures, several mAbs, including 2H12.G4 and 1H4.B6, stained the caveola of P. vivax CVCs in addition to the vesicles and tubules (Barnwell et al., 1990; Matsumoto et al., 1988). Future analysis of the iRBCs in different stages of development should prove to be informative with regards to the definitive static and dynamic localization of this protein. It is certainly possible, and cannot be excluded at this time, that the protein becomes localized to and functions at the cytoplasmic interface of the CVCs, and also within the caveolae.

Different numbers and types of PHIST family members (PHISTA, PHISTB, and/or PHISTC) with presumptive common ancestral lineages have been revealed in P. falciparum, P. vivax, P. knowlesi, P. gallinaceum, P. yoelii, P. berghei, and P. chabaudi and yet neither definitive localizations nor functions have yet to be ascribed to any members. Family members can differ considerably, for example by size, structure or transcription pattern, but they all have the so-called PHIST domain in common with its characteristic multiple alpha helices and conserved tryptophan residues (Sargeant et al., 2006). The presence of this domain defines family members, yet specific functions remain unknown (Sargeant et al., 2006). There have been 71 phist paralogs annotated in P. falciparum, 39 in P. vivax (18 paralogs reported in (Carlton et al., 2008)) and 27 in P. knowlesi (Sargeant et al., 2006); several PHIST proteins have also been identified in P. cynomolgi proteomic studies (Akinyi, Korir and Galinski, unpublished data). It remains to be determined whether all genes representing members of the PHIST family are expressed in each of these species, at what stage(s), and whether the different family members have quite different or similar functions. Our BLAST searches
identified which *phist* gene family member in other *Plasmodium* species is most closely related to *pvphist/cvc-8195* and *pcyphist/cvc-8195*, and the comparative strong homology of this selected gene set in the 5′ and 3′ regions encoding the N- and C-terminal sequences supports their proposed relationship as orthologs. Our analyses also show that each of these genes encodes a central domain of degenerate amino acid repeated motifs, or remnants thereof (Fig. S3). The repeated motifs are evident in the *P. falciparum, P. knowlesi, P. yoelii* and *P. berghei* PHIST homologs, whereas only apparent ancestral remnants exist in in the PvPHIST/CVC-8195 and PcyPHIST/CVC-8195 sequences, raising the possibility that the repeat motifs per se are not functionally important for these proteins. To the contrary, PvPHIST/CVC-8195 and PcyPHIST/CVC-8195 may have evolved to harness amino acid sequences that are critical for the structure and/or function of these proteins. Repeated motifs have not been previously noted as a main characteristic of PHIST family members, aside from being mentioned in one brief report (Chookajorn et al., 2006). Such variable and degenerate repeated motifs are not unique to PHIST, but have become recognized as typical features of various *Plasmodium* genes and proteins (reviewed in Anders et al., 1993) and their intra-species sequence diversity can be high in the midst of otherwise conserved sequences (e.g. see Galinski *et al.*, 1987; Cowman *et al.*, 1985).

*P. falciparum, P. knowlesi, P. yoelii and P. berghei* do not produce CVCs in their host iRBC membranes, and thus the respective PHIST homologs could reasonably be expected to perform a function(s) that may be quite different from PvPHIST/CVC-8195 and PcyPHIST/CVC-8195. It is worth noting in this regard, that PPHIST-147 (gene ID: Pf08_0137), was found to be associated with the *P. falciparum* translocon of exported proteins (PTEX) complex (de Koning-Ward *et al.*, 2009). The PTEX complex has been predicted to transport proteins from the parasite across the PV membrane to the erythrocyte cytosol. It is possible that homologous proteins, such as PHIST/CVC-8195, with conserved PHIST domains, may have different localizations in the course of development in each species yet share common primary functions, for example, serving as protein chaperones.

*P. cynomolgi* as an experimental model lends itself well to exploration of the many open questions relating to the biogenesis and function of the CVCs in the biology of *P. vivax*. For example, are the caveolae and tubules of the CVCs created as a result of the invagination of the iRBC membrane at numerous points? Or, do the CVCs develop through de novo biogenesis from a composite of proteins, lipids and vesicles through a process of progressive or pre-formed docking at the inner surface of the infected erythrocyte membrane? The ring stage IFA patterns representing PHIST are intriguing and distinctive compared to the later stage parasites (Fig. 3A). The “soccer ball” pattern of PHIST reactivity in these ring-stage IFAs is suggestive of a semi-organized initial placement of the CVCs at the surface of the iRBCs. If this pattern is indeed biologically relevant, perhaps the parasite requires an organized scaffold of selectively placed CVCs to brace the iRBC before progressively continuing the process of destroying and rebuilding the RBC natural cytoskeletal architecture to accommodate an increasing number of CVCs.

While some weak and limited *in vitro* cytoadherent characteristics have been noted for *P. vivax* iRBCs (Carvalho *et al.*, 2010), to date there is no evidence of a dominant cytoadhesion phenotype for *P. vivax* or *P. cynomolgi*, akin to that observed for *P. falciparum* and attributed to the adhesion of the parasite’s iRBC knobs with a variety of host endothelial receptors. *P. vivax* has no paralogs of the *var* genes of *P. falciparum*, known to encode the highly cytoadherent variant antigens (Scherf *et al.*, 2008), nor the related *SICAVar* genes of *P. knowlesi* (al-Khedery *et al.*, 1999; Korir *et al.*, 2006), but CVC could be a conduit for implanting members of the variable *P. vivax vir* gene family (Fernandez-Becerra *et al.*, 2005) or other functional proteins into the membrane of the iRBC. That CVC participate in or regulate some necessary metabolic or physiological functions seem more likely as the
primary purpose of these organelles; also making them strong potential targets of chemical or immune intervention. By comparison, Hanssen and colleagues reported 25 nm and 80 nm diameter vesicles in *P. falciparum* iRBC (Hanssen et al., 2008; Hanssen et al., 2010). The 25 nm vesicles were limited by an ordinary lipid bilayer and by ET appeared to be free in the cytoplasm or associated with Maurer’s clefts and the iRBC membrane. In contrast, the 80 nm vesicles had an electron-dense coat covering a 25 nm bilayer circumscribed aperture and were typically very few in number. While electron dense ‘knob’ protrusions are the predominant feature at the surface of *P. falciparum* iRBCs, in rare instances the 80 nm vesicles appear to have fused to the iRBC membrane to form caveolae, with a similar electron dense coat as observed for the caveolae component of the CVCs. Sparse caveolae have also been observed by TEM at the surface of *P. knowlesi* iRBCs (Aikawa et al., 1975). We envision that these caveolae may have a function distinct from that of the numerous CVCs found in *P. vivax* and *P. cynomolgi*.

Importantly, our replica in vivo transfection experiments support the hypothesis that the predominant 95 kDa CVC protein in *P. vivax* and *P. cynomolgi*, now known as PvPHIST/CVC-81 and PcyPHIST/CVC-81, is essential for parasite survival. *P. cynomolgi* parasites that had been transfected with a construct targeting the disruption of the pcyphist/cvc-81 gene were selected in vivo in rhesus monkeys, but only parasites containing episomes with the mutated tgdhfr gene conferring pyrimethamine resistance survived. Our parallel experiment showing that a transgene can in fact become integrated into the *P. cynomolgi* genome is particularly important, as this is the first evidence that this is indeed possible in this species. We have concluded at this stage, that the PHIST/CVC-81 protein is essential for the parasite. The precise roles this protein plays remains to be defined, but its abundance and prominent location suggests a critical role.

Aside from PHIST/CVC-81, a few other proteins have been defined in association with CVCs through either IFA or IEM. These include a large (>200 kDa) transmembrane protein (Barnwell and Galinski, unpublished data), a 70 kDa protein (Barnwell et al 1990) and an 86 kDa protein (Udagama et al., 1988). Caveolins and flotillin-2 have also been reported to be present within the CVCs by antibody reactivity (Bracho et al 2006). In future investigations it will be important to determine the full composite of proteins associated with the CVCs, define how the CVC architecture develops during the growth of the intra-erythrocytic parasite, and identify biological and biochemical processes they serve for the parasitized cell. Given the potential in medicinal chemistry to design novel drug compounds based on structure-associated relationships, further exploration in this direction seems highly warranted to develop new drugs active against *P. vivax*.

**Experimental Procedures**

**Parasite materials**

Using standard procedures, cryopreserved and reconstituted *P. cynomolgi* (Berok strain) ring iRBCs were inoculated as required into splenectomized *Macaca mulatta* (rhesus) monkeys following approved protocols from the Institutional Animal Care and Use Committee at Emory University. At an approximate 5% target parasitemia, blood was collected and passed through glass beads and cellulose CF11 columns to remove platelets and white blood cells, respectively, and then enriched for trophozoite-iRBCs by centrifugation over a 52% or 48% Percoll cushion, respectively, as described (Barnwell et al., 1990; Galinski et al., 1992).

*P. cynomolgi* genomic DNA (gDNA) was prepared from schizont-stage parasites using the QiAamp DNA Blood Extraction kit (Qiagen) following the manufacturer’s instructions. The schizonts were obtained from fresh infected blood, as described above, or from reconstituted
ring-stage iRBCs that were matured to the schizont stage in short-term culture as described (Barnwell et al., 1999).

Monoclonal antibodies

Monoclonal antibodies 2H8.E10, 2H12.B4, 4C12.G4 and 1H4.B6 were among a battery of mAbs raised against P. vivax schizont and merozoite proteins and characterized as previously described (Barnwell et al., 1990; Matsumoto et al., 1988; Barnwell, 1986). Total IgG was then purified using the Protein A MAPS affinity isolation system (BioRad) following the manufacturer’s protocol.

Production of recombinant protein and antisera

Pvphist/cvc-8195 gene-specific primers PvPHIST/CVC-8195F (5′ tat gga tcc ATG AGT CCC TGC AAC ATC) and PvPHIST/CVC-8195R (5′ ata ctc gag TTA GAG TTT GCT GTG TTT CT) were used to amplify the full length of the gene under standard PCR conditions following the manufacturer’s protocol (Calbiochem). The amplicon was cloned into the expression vector pGEX 4T-2 (GE Healthcare) using the BamHI and XhoI restriction sites. Positive clones were confirmed with an ABI 3100 DNA sequencer. The clones were then transformed into E. coli BL21 StarTM (DE3) cells (Invitrogen) for protein expression. Soluble protein was purified using Glutathione SepharoseTM 4B (GE Healthcare) slurry according to the manufacturer’s protocol. Recombinant PvPHIST-8195 (rPvPHIST/ CVC-8195) was inoculated into a New Zealand White Rabbit (Covance) for production of a polyclonal antiserum, rabbit anti-rPvPHIST/CVC-8195.

Indirect Immunofluorescence Assays (IFA) and western immunoblots

The cross-reactivity of the mAbs raised against P. vivax was tested by IFA on air-dried, cold acetone-fixed thin films of RBCs infected with P. cynomolgi ring, trophozoite or schizont stage parasites. Following incubation with the primary antibodies, expression was detected using affinity-purified goat IgG anti-mouse conjugated to Alexa Fluor 488 (Invitrogen) as secondary antibodies. The mAbs were tested at 1:100, 1:200, 1:400 and 1:800 dilutions in phosphate-buffered saline (PBS; Lonza) containing 0.2% bovine serum albumin (BSA; Sigma). The rabbit anti-rPvPHIST/CVC-8195 IFA reactivity was tested at dilutions of 1:100, 1:200, 1:400 and 1:800, followed by anti-rabbit antibodies conjugated to Alexa Fluor 488 (Invitrogen) at a 1:200 dilution. The parasite nuclei were visualized with DAPI, contained in ProLong Gold Antifade Reagent (Invitrogen). The slides were examined with a Zeiss Imager.Z1 or Axioskope 2 microscope with filters appropriate for the fluorescent dyes, and the images merged.

For western immunoblot analyses, P. cynomolgi trophozoite extracts were electrophoretically separated on SDS-polyacrylamide gels and then transferred to a 0.2 μm nitrocellulose membrane (Schleicher & Schuell) and probed with the mAbs diluted 1:1000. Membranes were incubated with the corresponding alkaline phosphatase-conjugate as a secondary antibody (Promega) and immunoreactivity was detected by incubating with NBT/BCIP substrate (Promega).

Immunoprecipitation of P. cynomolgi extracts with monoclonal antibodies

P. cynomolgi-iRBC extracts were prepared as follows: Ice-cold 1X NET/1% NP-40 containing protease inhibitors (10 mM EDTA-Na2; 1 mM PMSF; 0.1 mM each of TPCK, TLCK, Leupeptin, Chymostatin, Antipain and 3,4-DCI; 10 μM EP-64 and 1 μM Pepstatin A; Sigma) was added to P. cynomolgi-infected erythrocyte pellet, and extracted for 30 min on ice with occasional vortexing. The samples were then transferred to pre-chilled microcentrifuge tubes and spun at 20,817 × g for 20 min at 4°C. The resulting NP-40
extracts were transferred and stored, while the pellet was extracted in 1% SDS by occasional vortexing for 10 min at room temperature. The sample was then centrifuged at 20,817 × g for 20 min at room temperature. The SDS extracts were combined with the NP-40 extracts for use in immunoprecipitations.

One hundred sixty μl of rProtein G agarose suspension (Invitrogen) were incubated with 200 μg of each P. vivax mAb overnight at 4°C, rotating. Meanwhile, extracts were preclarified with rProtein G agarose overnight at 4°C, on a rotational shaker. The extracts were then separated from the preclarifying beads. Dimethyl pimelimidate (Sigma) was added to the mAb/rProtein G mix to a final concentration of 20 mM and incubated for 6 h, at 4°C, rotating. The uncoupled mAb was then removed by pulse spin, and the mixture washed with NETT (150 mM NaCl/5 mM EDTA/50 mM Tris/0.5% Triton X-100). One milliliter of the combined extract was added to the coupled mAb/rProtein G mix and incubated overnight. The beads were then washed twice with NETT, twice with NETT/0.5 M NaCl and once with NETT/0.05% SDS. The samples were resuspended in 2X SDS-PAGE loading buffer and resolved on 4–20% gradient SDS-PAGE gels (Bio-Rad).

Mass spectrometric analysis of immunoprecipitated proteins

After resolving the extracts and membrane samples on 4–15% SDS-PAGE gradient gels, the gels were stained with colloidal Coomassie blue (Imperial Protein Stain; Thermo Scientific). Gel slices were then excised, destained, dried, and processed as described (Korir et al., 2006). Briefly, the gel pieces were digested with trypsin (Sigma) and the resulting peptides extracted with trifluoroacetic acid (Sigma). The samples were then desalted and concentrated using ZipTip pipette tips (Millipore). Cleaned peptides were analyzed by reverse-phase LC-MS/MS (Peng et al., 2001) using an LTQ-Orbitrap mass spectrometer (Thermo Finnigan). A reverse database strategy using the SEQUEST algorithm was implemented to evaluate false discovery rate; the matched peptides were filtered according to matching scores to remove all false matches from the reverse database (Peng et al., 2003). Only proteins that were matched by at least two peptides were accepted to further improve the confidence of identification. The peptides were then searched against the NCBI database, with searches being limited to Plasmodium results.

Bioinformatics for peptide and gene analyses

Protein matches acquired from LC-MS/MS were searched against Plasmodium (PlasmoDB; www.plasmodb.org) and general (National Center for Biotechnology and Information; http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein&itool=toolbar) databases to determine the homologous genes. Signal peptide cleavage sites and transmembrane domains were predicted with SignalP V3.0 (http://www.cbs.dtu.dk/services/SignalP/) and TMpred (www.ch.embnet.org/software/TMPRED_form.html) software, respectively. Multiple alignments of PHIST protein sequences were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) or the MacVector v7.2.3 software. The GOR4 program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html; Combet et al., 2000) was used to predict secondary structure using the amino acid sequences of the PHIST family members.

Polymerase Chain Reaction amplification and cloning of the pcyphist/cvc-8195 gene

Polymerase Chain Reaction (PCR) amplification of the pcyphist/cvc-8195 gene from P. cynomolgi gDNA was performed using a combination of pvphist-8195 and the related pkphist-105 (Fig. 2, S1, S2) gene-specific primers and the Expand High Fidelity System (Roche) kit as per the manufacturer’s protocol. PCR products were then purified using the Qiaquick purification system (Qiagen), cloned into the pCR2.1 vector (Invitrogen) and sequenced using the ABI Prism BigDye Terminator v3.0 cycle sequencing kit (Applied
Biosystems). By gene walking, and the subsequent design of pcyphist/cvc-8195-specific primers, the first 2,200 nucleotides of the pcyphist/cvc-8195 gene were sequenced and verified. To sequence the 3′ end of pcyphist/cvc-8195 up to the stop codon, degenerate reverse primers were designed based on the 3′ UTR sequences of pvphist/cvc-81 and pkphist-105. The primer pair PcyPHIST/CVC.2023.F (5′ GAT GCA AGA GTA CAT TAT GC) and PcyPHIST/CVC.3′ UTR.R (5′ CCA AA(A/C) GTT CTC CTA TGA CG) amplified the sequence up to the stop codon. This result was confirmed by sequencing the entire gene using pcyphist/cvcet8195-specific primers:

PCyPHIST/CVC.1.F (5′ ATG AGT CCC TGC AAC ATC),
PCyPHIST/CVC.263.R (5′ CTC AGA GAG ATA TGC TCA AA),
PCyPHIST/CVC.566.R (5′ CAT CTC CTC TCG TTG CCA),
PCyPHIST/CVC.762R (5′ TCA GAG GGA TCG GTA TCG),
PCyPHIST/CVC.1229.R (5′ CAC CTC TTC CGT GGT ATT),
PcyPHIST/CVC.2023.F (5′ GAT GCA AGA GTA CAT TAT GC),
PcyPHIST/CVC.2160.R (5′ GAG TAT TGC ATA ATG TAC TC), and
PcyPHIST/CVC.2433.R (5′ TAC AAT TTA CTG TGT TTC TTC).

Transfection of *P. cynomolgi* iRBCs

Selection Cassette—A vector with a pBlueScript backbone containing a pyrimethamine selection cassette inserted into the NdeI restriction site of the multiple cloning site was kindly provided by Alan Thomas (van der Wel et al., 1997). This vector includes the following gene fragments positioned head to tail: 5′ UTR sequence of pbdhfr-ts, mutated tgdhfr-ts coding sequence, and 3′ UTR sequence of pbdhfr-ts. Its total size is 4,996 bp. Three mutations of the tgdhfr-ts coding sequence (Ser_36Arg, Thr_83Asn and Phe_245Ser) required to confer resistance to pyrimethamine were confirmed by end sequencing.

*P. cynomolgi* phist/cvc-8195 knockout construct (pcyΔphist/cvc8195 vector)—The following primer pairs were designed to amplify 600 bp from the 5′ region of pyphist/cvc-8195 (from −555 to 45) and 554 bp from the 3′ region of the pcyphist/cvc-8195 (from 693 to 1246): Pcyphist/cvc-8195 3′ FP, ATTT CCCGGG AGAATGTATGATGAAGAATA; Pcyphist/cvc-8195 3′ RP, TCCATATCAAGTCTTCCACCTCGT; Pcyphist/cvc-8195 5′ FP, TTAAGAGAGCCATCGATGCC; and Pcyphist/cvc-8195 5′ RP, ATTT CCCGGG ATCATAGTAGTCATGGTTAC. The SmaI site required for subsequent reactions is underlined. All amplicons were generated using the KOD polymerase (Novagen). The 5′ and 3′ fragments were ligated using the SmaI restriction site added to the reverse primer of the 5′ region and the forward primer of the 3′ region, and amplified again with the forward primer recognizing the 5′ region and reverse primer recognizing the 3′ region. The amplicon representing the combined 5′ and 3′ fragments was inserted into the SmaI site of the pUC19 vector (New England Biolabs) to make the pUCpcyphist5′/3′ construct. Ligation of the selection cassette into the pUCpcyphist/cvc5′/3′ vector required the addition of a blunt restriction enzyme site to the selection cassette. Briefly, a 24 bp adaptor carrying the Pmel/NdeI/Pmel restriction site sequence was inserted into the EcoRV restriction site of pCR2.1. The selection cassette was released from the pBS vector using NdeI and then subcloned into the NdeI site of the pCR2.1 Pmel/NdeI/Pmel sequences so that the cassette could be released by digestion with Pmel. The final vector was generated by blunt end ligation of the Pmel rekaased selection cassette into the pUC19 pcyphist/cvc5′/3′ plasmid digested with SmaI. In vivo selection. Sequential transfection experiments were carried out,

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with the animal experimental protocols presented here approved by Emory University’s Institutional Animal Care and Use Committee.

For each experiment, a donor rhesus macaque was inoculated with ~1.5 \times 10^8 \textit{P. cynomolgi} (Berok) ring-stage iRBCs. A blood sample was drawn when the parasitemia reached >4% with a majority of the iRBCs at the schizont stage. The infected blood was processed using standard procedures (Barnwell \textit{et al.}, 1999) and mature schizonts were then separated using a Percoll (Amersham) gradient of 50%, washed in RPMI and resuspended in cytomix (Kocken \textit{et al.}, 1999) at 1.5 \times 10^9 parasites ml\(^{-1}\). Electroporation was performed with a BioRad Gene pulser II (25 \mu F, 200 Ohms and 2.5 kV (Kocken \textit{et al.}, 1999; van der Wel \textit{et al.}, 1997) with 0.4 ml of an iRBC suspension mixed with 0.4 ml of linearized plasmid DNA (0.1 mg) in a 0.4 cm cuvette at room temperature. The electroporated iRBCs were resuspended in RPMI and inoculated immediately into a recipient rhesus macaque. Parasitemias were checked daily by microscopic examination of blood smears. Once the parasitemia was rising, doses of 1 mg kg\(^{-1}\) of pyrimethamine (Sigma) were administered, with dosing times as noted in the results section. Once the pyrimethamine resistant parasitemia was rising to >3% infected blood was collected and cryopreserved using standard procedures. The animals were then treated with a standard curative regimen of chloroquine (15mg kg\(^{-1}\) 1M × 3 days) to terminate the infections.

Transmission electron microscopy and immuno-labeling

\textit{P. cynomolgi} trophozoite-iRBCs were fixed in incomplete RPMI-1640 containing 2% paraformaldehyde (PFA) and then permeabilized with EqtII (Anderluh \textit{et al.}, 1996) as previously described (Jackson \textit{et al.}, 2007, Hanssen \textit{et al.}, 2008). The samples were refixed in PBS containing 2% paraformaldehyde, blocked with 3% BSA in PBS and then incubated with rabbit anti-rPvPHIST-81\(_{95}\) as a primary antibody in PBS/3% BSA. After washing, the cells were incubated with 6 nm gold-conjugated Protein A (Aurion) according to the manufacturer’s instructions, washed again, and then fixed with 1% glutaraldehyde/0.5% PFA/0.1 M cacodylate buffer overnight. The samples were then embedded in 3% agarose and rinsed with 0.175 M cacodylate buffer. After post-fixation with 1% osmium tetroxide, the cells were stained ‘en-bloc’ with 1% uranyl acetate, and then serially dehydrated and embedded in LR White resin. The samples were sectioned to 70 nm thickness and after staining with lead citrate and uranyl acetate, observed at 120kV on a 2010HC (Jeol, Japan) transmission electron microscope (at La Trobe University EM Facility, Melbourne).

Electron tomography

Tomography was performed as described previously (Hanssen \textit{et al.}, 2008). 200–300 nm sections were cut and collected on a grid and then incubated with fiducial gold particles. The sections were then contrasted with lead citrate and uranyl acetate and observed on a tilt series from −69 degrees to 69 degrees at every 1.5 degrees between captured images for the first axis and every 3 degrees between captured images for the second axis. Data were acquired at an accelerating voltage of 200 kV using a Tecnai G2 TF30 transmission electron microscope (FEI, The Netherlands) at the Bio21 Institute electron microscopy facility (Melbourne). The tilt images were aligned and tomograms generated and rendered using the IMOD package (Kremer \textit{et al.}, 1996).

Nucleotide sequence accession number

The genomic sequence of \textit{pcyphist-81}_{95} was submitted to the GenBank database under accession number JN636815.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


Fig. 1.
The predominant 95 kDa CVC protein is a member of the PHIST protein superfamily. A. Air-dried, acetone-fixed smears of *P. cynomolgi* trophozoite-iRBCs incubated with the nuclear DAPI stain (blue) and *P. vivax* mAbs 4C12.G4 (1), 2H8.E10 (2), 2H12.B4 (3) and 1H4.B6 (4) followed by Alexafluor 488-conjugated goat anti-mouse IgG (Invitrogen) (green). Immunofluorescence was detected using a Zeiss Imager.Z1 microscope (panels 1–3), and Zeiss Axioskop 2 (panel 4). Scale bar: 4 μm. B. *P. cynomolgi* trophozoite-iRBC extracts were separated by SDS-PAGE under reducing conditions on a 4–20% gradient gel, transferred to a nitrocellulose membrane, and probed with *P. vivax* mAbs 2H12.B4, 4C12.G4, 2H8.E10 and 1H4.B6. A characteristic band of 95 kD (Barnwell *et al.*, 1990) was consistently obtained with all four mAbs (lanes 1–4), and a predominant breakdown product at 75 kDa with some extracts (lanes 3 and 4). Lanes 1 and 2, and 3 and 4 represent different extracts. C. LC-MS/MS was performed on bands excised from 4–20% SDS-PAGE gels containing proteins from *P. cynomolgi* trophozoite-iRBC extracts immunoprecipitated with mAbs 2H12.B4, 4C12.G4, 2H8.E10 and 1H4.B6; a representative experiment using mAb 1H4.B6 is shown. The proteins identified by LC-MS/MS are noted as PHIST/CVC-81 or IgG.
Fig. 2.
Protein structure and sequence identity of PcyPHIST/CVC-8195 and its homologs. A. The schematic represents the PvPHIST/CVC-8195, PcyPHIST/CVC-8195, PkPHIST-105 and PfPHIST-147 proteins, showing their number of amino acids and main features as indicated. B and C. N- and C-terminal amino acid sequence alignments generated using ClustalW. Positions of identity across all four species are shown in dark gray; the hydrophobic sequence is marked by a brown line; the putative PEXEL motif is boxed in red; and the conserved tryptophans are denoted with a green triangle. The four regions predicted using the GOR4 algorithm (Combet et al., 2000) to form alpha helices are shown within the blue boxes.
Fig. 3.
PcyPHIST/CVC-81\textsubscript{95} is expressed in the ring, trophozoite and schizont stages of development.

A. IFA tests. Air dried and acetone-fixed smears of ring, trophozoite and schizont-stage \textit{P. cynomolgi}-iRBCs were incubated with rabbit anti-rPvPHIST/CVC-81\textsubscript{95} serum and mounted in Prolong Gold Antifade Reagent containing the nuclear DAPI stain (Invitrogen; blue). Positive antibody reactivity was detected using Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, green). The ring-stage panel illustrates a “soccer ball” pattern of PHIST reactivity; white arrows point to blackened areas lacking fluorescence. Immunofluorescence was detected using a Zeiss Imager.Z1 microscope. Scale bar: 0.4\textmu m.

B. Western immunoblot. SDS detergent extracts from $1 \times 10^8$ \textit{P. cynomolgi} ring, trophozoite and schizont stage iRBC pellets were diluted 1:100 in sample buffer and subjected to SDS-PAGE under non-reducing conditions on a 4–20% gradient gel. Rabbit anti-rPvPHIST/CVC-81\textsubscript{95} was used at a 1:1000 dilution.
Fig 4.
Transmission EM of intact and permeabilized *P. cynomolgi* iRBCs. A. An intact iRBC showing one CVC at the surface (arrow); note the presence of a mitochondrion (m). B. An EqtII permeabilized iRBC section showing one CVC with several long extensions. C. Low magnification image showing a section of a whole EqtII permeabilized iRBC showing the presence of multiple CVCs (several indicated with arrows), clefts (C) and parasite (P). D-F. Immunolabeling (denoted with arrowheads) of CVCs in EqtII permeabilized iRBCs using mAb 4C12.G4 (D), rabbit anti-rPvPHIST/CVC-8195 (E) and a negative control (F). Scale bars: 200 nm.
Fig 5.
Electron tomography of CVCs in a *P. cynomolgi* trophozoite-iRBC. Virtual sections through an approximately 200 nm thick tomogram are presented. Each image represents a thickness of 2.7 nm and the spacing between each image is 18 nm. *P. cynomolgi* trophozoite-iRBCs were permeabilized with EqtII and prepared for electron tomography. A dual tilt series was collected every 1.5° for the first axis and every 3° for the second axis, and used to generate a 3-D volume. Caveolar openings are indicated with arrowheads. The RBC membrane is indicated with an open arrow, and isolated vesicles with diameters ranging from 25 – 45 nm are noted with black arrows. The sequence of images is from the top right to bottom left, as indicated by a directional arrow in the top right panel. Scale bar 200 nm.
Fig. 6.
Immuno-electron tomography of a CVC in a *P. cynomolgi* trophozoite-iRBC. A–H. Equinatoxin II permeabilised *P. cynomolgi* trophozoite-iRBCs were labeled with two different antibodies (A–D, mAb 4C12.G4 and E–H, rabbit anti-rPvPHIST/CVC-8195). A–C and E–G. Selected virtual sections (20 nm) showing CVCs. D and H. Segmentation models were created with IMOD software. The RBC membrane is rendered in blue, the CVC in orange, and gold particles in yellow. The CVC opening on the surface of the iRBC is indicated by thick arrows (A,D,F,H). A long tubular extension is highlighted by an arrowhead in B and the same extension is shown with an arrowhead in D. A section through one of the short tubular extensions is highlighted by an arrowhead (G) and the same extension is shown with an arrowhead in H. The gold particles are concentrated on the tubular extensions as denoted with fine arrows (D,H). Bar: 100 nm.
Fig. 7.
A. *P. cynomolgi* phist/cvc-81<sub>95</sub> transfection experiments result in retrieval of episomes conferring resistance to pyrimethamine, but without integration in the genome. Results are shown for one of two transfection experiments. A. Schematic of the pcyphist/cvc-81<sub>95</sub> target gene (top) and the related gene disruption vector construct with a pUC-19 backbone (bottom). Red arrows represent the location of phist/cvc forward (FP) and reverse (RP) oligonucleotide primers used in diagnostic PCR amplifications. The hatched boxes represent the 5′ and 3′ ends of the pcyphist/cvc-81<sub>95</sub> gene. The pcyphist/cvc-81<sub>95</sub> sequences flank the selection cassette, comprised of the *P. berghei* 5′ and 3′ UTRs and the mutated tgdhfr-ts gene to confer resistance to pyrimethamine. EcoR1 (R1) and BamHI (HI) restriction enzyme sites were used to linearize this construct and release the selection cassette for transfection experiments. B. Using phist/cvc-FP and phist/cvc-RP, the pcyphist/cvc-81<sub>95</sub> gene was amplified by PCR from genomic DNA purified from both wild type (Wt) and transfected (Tx) parasites. C. Plasmid DNA bands of 5.1 kb and 3.6 kb corresponding to the gene disruption vector were retrieved after *in vivo* selection in rhesus monkeys, purification of DNA from selected parasites, subsequent transformation of *E. coli*, and digestion with R1 and EcoRV (RV) restriction enzymes. D. IFA experiments show a normal speckled expression pattern for PHIST/CVC in Tx parasites. *P. cynomolgi* trophozoite-iRBCs are shown with rabbit anti-PHIST/CVC-81<sub>95</sub> reactivity (green) and DAPI nuclear stain (blue). Immunofluorescence was detected using a Zeiss Imager.Z1 microscope. Scale bar: 4 μm. E. Western immunoblot showing rabbit anti-PHIST/CVC-81<sub>95</sub> reactivity (95 kDa, arrow, with associated breakdown products) on both Wt and Tx trophozoite-iRBCs.
Table 1

Identity between PvPHIST/CVC-81₉₅ and PcyPHIST/CVC-81₉₅

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<thead>
<tr>
<th></th>
<th>Nucleotide</th>
<th>Amino acid</th>
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<tr>
<td>Global</td>
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