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Recruitment of Factor H as a Novel Complement Evasion Strategy for Blood-Stage Plasmodium falciparum Infection

Alexander T. Kennedy,*† Christoph Q. Schmidt,‡ Jennifer K. Thompson,* Greta E. Weiss,¶ Tana Taechalertpaisarn,* Paul R. Gilson,¶ Paul N. Barlow,¶,# Brendan S. Crabb,¶,*∥∥∥∥ Alan F. Cowman,*† and Wai-Hong Tham¶,*†

The human complement system is the frontline defense mechanism against invading pathogens. The coexistence of humans and microbes throughout evolution has produced ingenious molecular mechanisms by which microorganisms escape complement attack. A common evasion strategy used by diverse pathogens is the hijacking of soluble human complement regulators to their surfaces to afford protection from complement activation. One such host regulator is factor H (FH), which acts as a negative regulator of complement to protect host tissues from aberrant complement activation. In this report, we show that Plasmodium falciparum merozoites, the invasive form of the malaria parasites, actively recruit FH and its alternative spliced form FH-like protein 1 when exposed to human serum. We have mapped the binding site in FH that recognizes merozoites and identified Pf92, a molecule of the six-cysteine family of Plasmodium surface proteins, as its direct interaction partner. When bound to merozoites, FH retains cofactor activity, a key function that allows it to downregulate the alternative pathway of complement. In P. falciparum parasites that lack PF92, we observed changes in the pattern of C3b cleavage that are consistent with decreased regulation of complement activation. These results also show that recruitment of FH affords P. falciparum merozoites protection from complement-mediated lysis. Our study provides new insights on mechanisms of immune evasion of malaria parasites and highlights the important function of surface coat proteins in the interplay between complement regulation and successful infection of the host. The Journal of Immunology, 2016, 196: 000–000.

Malaria kills ~600,000 people each year and infects millions more worldwide (1). The malaria life cycle is complex involving both human and mosquito hosts. The bite of an infected Anopheles mosquito injects sporozoites, a motile form of the malaria parasite, into the human host. The sporozoites travel to the liver where they undergo rounds of replication in hepatocytes that eventually rupture releasing merozoites, the invasive form of the malaria parasite, into the bloodstream. The merozoites invade erythrocytes and replicate as trophozoites, which divide via schizogony to produce more merozoites perpetuating the infection. Release of merozoites from ruptured erythrocytes occurs every 48 h for Plasmodium falciparum, the species of human malaria that causes the majority of deaths. Blood-stage infection is responsible for the clinical symptoms of malaria. Once released, these merozoites are competent for invasion for a short period (2). For this brief period the parasite is no longer hidden within its host cells and is thus exposed to the surveillance of the host’s immune system.

For example, merozoites must avoid destruction by the complement system, the frontline defense against invading pathogens. The complement system includes a cascade of sequentially activatedzymogens that brings about opsonophagocytosis or membrane lysis of the invading organism (3). Complement activation occurs via the classical pathway (CP), lectin pathway (LP), or alternative pathway (AP) (4). The CP is triggered by Ab-Ag complexes, whereas the LP is initiated by recognition of nonself carbohydrates (4). The AP is constitutively active and is driven by low levels of backgroundzymogen activation (5, 6).

The CP, LP, and AP each induce assembly of C3 convertases that cleave complement component C3 to C3b, exposing a thioester domain that rapidly undergoes covalent linkage to hydroxyl or amine groups (7). In this way, C3b can become tethered to nearby surfaces. On a foreign surface, C3b can bind factor B that is then cleaved, forming C3bBb. This is the C3 convertase, able to produce additional C3b (6), thus creating a positive feedback loop that can cause an entire bacterial cell with C3b molecules within 10 min. Once the density of C3b molecules on the surface exceeds a critical threshold, C5 convertases form that cleave complement component C5 to C5a and C5b. C5b in turn recruits C6, C7, C8, and C9 to form the deadly membrane attack complex (MAC), resulting in damage or lysis of the targeted cell (8).
C3b amplification is prevented on self-surfaces by a family of proteins called regulators of complement activation to prevent destruction. One of the major complement regulators of the AP is factor H (FH). This 155-kDa protein is a key soluble regulator of the AP and of the C3b amplification loop. Operating in both fluid phase and on self-surfaces, it is composed of 20 complement control protein modules (CCPs) each containing ~60 aa residues (9). Domains 1–4 of FH confer its regulatory properties, which include accelerating C3 convertase decay, cofactor activity for the protease Factor I facilitating cleavage of C3b to iC3b (which no longer is able to form convertases), and finally inhibition of the initial formation of the AP C3 convertase C3bBb by competing with Factor B for binding to C3b. FH domains 6–8 and 19–20 have a polyanion binding site facilitating recognition of self-surfaces, whereas domains 19–20 also contain a C3b binding site (10–13). The combination of C3b and polyanion binding at the C terminus allows FH to preferentially bind to self-surfaces that are under complement duress (14, 15). FH-like 1 (FHL-1) is an alternative spliced product of FH, found in serum, at about half the molar concentration of FH (16, 17). FHL-1 consists of the first seven CCPs of FH, as well as four additional C-terminal amino acids, and has similar regulatory activities as FH but lack the self-surface recognition capabilities endowed by CCPs 19 and 20 of FH.

Many pathogens grow and thrive in serum despite exposure to complement. They have evolved mechanisms to evade complement-mediated clearance including the hijacking of human complement regulators (18). Several pathogens recruit FH to regulate complement activation on their surfaces: Neisseria meningitidis expresses FH-binding protein, which mimics the carbohydrate motif of FH would recognize on self-surfaces (19, 20); Borrelia spp. use the CRASP family of proteins, whereas the M-protein family are used by Streptococcus (21). Many of these FH-binding proteins are virulence factors (22, 23).

Plasmodium merozoites possess a densely packed surface coat of merozoite surface proteins (MSPs; reviewed in Ref. 24). These proteins may be attached to the parasite by a transmembrane domain, or more commonly a GPI anchor. Alternately, the proteins may peripherally associate with the surface by interactions with other proteins on the surface. One particular family of proteins, the six-cysteine (6-cys) family, has been implicated in immune modulation, in addition to other functions (25, 26). The 6-cys family is defined by domains containing six conserved cysteine residues and is unique to the Plasmodium species, which expresses multiple members of this family in different life stages (27–29). Merozoites express Pf12, Pf92, and Pf38 as GPI-anchored products, whereas Pf41 is peripherally associated (30–33).

Recent studies have highlighted how malaria parasites may have evolved mechanisms to cope with complement activation. Infected RBCs with late-stage parasites (schizonts) recruit FH onto their surfaces using an unknown trypsin-resistant parasite receptor (34). Inhibition of FH function using an inhibitory anti-FH mAb results in a 10% reduction in parasitemia in the presence of active serum. These results show that FH recruitment to infected RBCs is required to protect blood-stage parasites from complement destruction. Gametocytes, the sexual forms of the malaria parasite, emerge in the mosquito midgut where they are exposed to complement from the blood meal. It was reported that they use the parasite protein GAP50 to recruit the human regulator FH onto their surface (35). Furthermore, inhibition of this recruitment reduces gametocyte survival, indicating its protective effect. In blood-stage infection, schizont rupture releases merozoites and digestive vacuoles leading to immune system exposure. A recent study has found digestive vacuoles serve as major complement activation targets with MAC forming on their surface (36–38). Intriguingly, these studies did not observe terminal pathway formation on merozoites, a strong indication that complement evasion strategies are in place to protect this cell type from complement-mediated attack.

In this work, we show that P. falciparum merozoites protect themselves from complement attack through recruitment of human complement regulator FH by one of their surface coat proteins and provide the first evidence, to our knowledge, for complement evasion in the blood stages of malaria infection.

### Materials and Methods

#### Parasite culturing

Parasites were maintained by serial passage in RPMI-HEPES containing 0.5% w/v Albumax II (Life Technologies), 2% hematocrit of O+ blood (Australian Red Cross Blood Bank). We used D10-PHG (39) for the majority of our assays, herein referred to as wild-type (WT). The strategy for generation of ΔPf92 is shown and described in Supplemental Fig. 4. Generation of ΔPf12 knockout is described by Taechalertpaisarn (33).

#### Complement deposition of merozoites

Merozoites were obtained as previously described (2). Merozoites were incubated in either normal human serum (NHS), heat-inactivated NHS (NHS-H; 56˚C 30 min), NHS inactivated by addition of EDTA to a final concentration of 10 mM (NHS-E), or a mixture of purified complement components at 37˚C. Complement activation was terminated by addition of protease inhibitor mixture with EDTA (Roche) followed by incubation on ice. After three washes, complement-deposited merozoite samples were used for further assays as described later.

#### Recombinant protein expression of FH fragments

Recombinant fragments of FH were produced using a Pichia pastoris expression system as described previously (13, 40). In brief, coding sequences for the recombinant forms of FH were amplified from cDNA or a codon-optimized FH gene and cloned into the P. pastoris pPICZαB expression vector (Invitrogen). Recombinant proteins were secreted into the medium and purified by successive ion exchange and size exclusion chromatography steps.

#### Abs

Abs to complement proteins FH and C3 were obtained from Complement Technologies. Abs to MSPs apical membrane Ag-1 (AMA1), Pf92, and Pf12 were generated at the Walter and Eliza Hall mAb facility.

#### Western blotting

Complement deposited merozoites were resuspended in reducing or non-reducing sample buffer and fractionated by SDS-PAGE before transfer onto nitrocellulose membranes (GE Healthcare Life Sciences). After blocking, membranes were incubated with primary Abs, washed, and then incubated with appropriate secondary Abs conjugated to either HRP or fluorophores before development.

#### Immunofluorescence assay

Immunofluorescence assays were performed as previously described (41). Primary Abs were incubated with cells for 1 h followed by PBS washes. Merozoites were subsequently labeled with secondary Abs (Alexa Fluor; 555 and 647 nm) and washed three times with 500 μL PBS. Slides containing Vectashield (Vector Labs) with 0.1 ng/μL DAPI were imaged using a DeltaVision Elite widefield fluorescence microscope (DeltaVision Technologies) with a 100× Olympus oil objective (Numerical aperture: 1.40) and images taken using Softworx (v.6.1; DeltaVision) on a Coolmap HQ2 camera (Photometrics) at ambient temperature. Softworx was used for enhanced ratio deconvolution before maximum projection and pseudocoloring with Fiji (ImageJ v2.0). Fiji was also used to generate merged images.

#### Flow cytometry of FH binding

Merozoites were purified as described earlier followed by digestive vacuole and hemozoin removal via passage over a magnetic column (MACS Miltenyi). Complement deposited merozoites were incubated with anti-FH for 30 min, washed, and then incubated for 30 min with EtBr (Bio-Rad) and
anti-goat 633 nm (Alexa Fluor). Samples were washed and analyzed using a FACSCalibur machine. Merozoites were gated based on positive GFP expression and EtBr+ staining as described previously (2, 42). FH-bound merozoites were GFP+ and 633 nm positive. Merozoites incubated only with anti-goat 633 nm secondary Ab served as the negative control for FH staining and gating. More than 50,000 events were collected before analysis with FlowJo V8.8.7.

**Complement destruction assay for merozoites**

Merozoites were purified as described earlier with digestive vacuoles and hemozoin removed and subsequently incubated with human serum. Merozoites were washed with PBS/1% w/v BSA, then stained with EtBr. Cells were washed and resuspended in PBS before analysis with FACSCalibur. Merozoites were gated as EtBr+ as described previously (2, 42). An unstained buffer control with merozoites acted as a control for gating cutoffs. More than 50,000 events were collected for each sample and analyzed using FlowJo V8.8.7.

**Cofactor activity assay**

FH, FHL-1, CCP1–4, or merozoites deposited with these regulators were mixed with 10 µg/ml C3b and Factor I (Complement Technologies) to a final volume of 20 µl for 2 h at 37°C. Factor I positive and negative controls were used to account for nonspecific cleavage events. Reducing sample buffer was added to solution phase assays. For merozoite preparations, the sample was centrifuged and supernatant was added to reducing sample buffer. Samples were separated via SDS-PAGE, and Western blotting analysis was performed using polyclonal anti-C3 Abs.

**Immunoprecipitation assays**

Saponin-treated parasites were solubilized overnight at 4°C using TNE buffer (1% v/v Triton-X 100, 150 mM NaCl, 10 mM EDTA, Tris pH 7.4). The supernatant was precipitated overnight with protein G-Sepharose beads. Purified FH or FH fragments were added to 30 mM final concentration or an equal volume of human tonicity PBS. Binding with protein G-Sepharose cross-linked (Thermo Scientific) to anti-FH, anti-Pf12, or anti-Pf92 was conducted before washing with TNE buffer before elution. For SDS-PAGE, beads were glycerol eluted for liquid chromatography–tandem mass spectrometry analysis. Elution used 125 mM TCEP, 25% v/v TFE in 0.1% v/v formic acid at 40°C with shaking. Mass spectrometry samples were neutralized by addition of TEAB, then trypsin digested overnight before liquid chromatography–tandem mass spectrometry.

**Parasite growth assays**

W2mef or W2mefAPf92 parasites were tightly synchronized and during the last cycle before the assay maintained in hirpan sulfate at 100 µg/ml to prevent invasions before commencement of the experiment. Heparin was removed and parasites were immediately transferred to RPMI 1640 with 30% final concentration of either active sera or heat-inactivated sera. Egress and invasion were allowed to proceed for 4 h with all assays done in triplicate or quadruplicate. Parasites were allowed to grow for two or four cycles, and at 32–36 h of age for each of these cycles the parasites were assayed to determine total parasitemia using the Malstat assay (43). Results are expressed as the percent of parasitemia relative to the heat-inactivated sera control for each sample in each assay, which was arbitrarily set at 100%. Statistical analysis was done using GraphPad Prism and a paired t test was used, pairing averaged values for each experiment done in triplicate or quadruplicate as compared with repeats of each experiment.

**Statistical analyses**

Statistical analyses were conducted using Prism for Mac version 6.0f (GraphPad Software, La Jolla, CA) for our flow cytometry analyses of FH binding from serum (Fig. 1D) and recombinant FH fragment binding (Fig. 2D). We used a one-way ANOVA with multiple comparisons between the means of each group from three independent experiments to test whether there was a difference in percentage of FH+ binding for each condition. For our merozoite destruction assay (Fig. 6A, 6B), we used STATATA v12.1 (StataCorp, College Station, TX) to perform two-way ANOVA analyses to test whether a difference existed in the percentage of EtBr+ events between serum conditions (NHS versus NHS-HI) and concentrations (10 versus 100%). We also checked for interaction effects between these two conditions and observed no significant interaction effects. Subsequently, we tested whether a difference existed between 100% NHS and NHS-HI, as well as 10% NHS and NHS-HI, using a one-way ANOVA (Fig. 6A, 6B). For Fig. 6C, to determine whether the loss of FH binding capacity caused a significant change in complement susceptibility, we divided the number of EtBr+ merozoites in NHS-HI by those in NHS to obtain a fold-change. A two-way ANOVA test was then used to compare whether a difference existed in fold-change...
FIGURE 2. Merozoites recruit FH and FHL-1 to their surfaces using CCP 4–6. (A) Schematic of the 20 CCP domains (gray circles) that represent FH. Green bars beneath the schematic span the different recombinant FH fragments, and the numbers on the right-hand side refer to the CCP domains that are within them. (B) SDS-PAGE of 4 μg of each FH recombinant fragment shown on schematic above and stained with Coomassie brilliant blue. Molecular mass marker in kDa is displayed on the left-hand side. (C) Merozoites were deposited with 0.5 μM purified FH and 0.5 μM recombinant FH fragments in buffer. After washing, eluates from merozoite pellets were fractionated by SDS-PAGE and analyzed by Western blotting with polyclonal anti-FH to identify regions that bind to merozoites. The membrane was reprobed with anti-AMA1 as a loading control. Molecular mass marker is displayed to the left in kDa. Lane names correspond to the CCP fragments indicated in (A). (D) Merozoites were deposited with 0.5 μM FH, FHL-1, and other recombinant fragments. After labeling with polyclonal goat anti-FH and anti-goat conjugated to Alexa 633, FH+ merozoites were identified using flow cytometry. Error bars indicate SEM from three independent experiments. All statistics are one-way ANOVA. *p < 0.05 compared with FH, #p < 0.05 compared with FH, FHL1, and 6–8; n.s., nonsignificant compared with FH. (E) A selection of recombinant FH fragments was deposited onto merozoites at 0.5 μM concentration in buffer. Merozoites were subsequently fixed and FH binding was detected with anti-FH Abs followed by anti-goat Alexa 647 secondary (green). Abs to AMA1 followed by anti-mouse conjugated to Alexa 555 were used to demarcate the merozoite surface (magenta). DAPI was included at 0.1 ng/μl in Vectashield to stain the merozoite nuclei (cyan). Images were captured on a DeltaVision Elite widefield fluorescence microscope using an Olympus 100× oil objective (NA: 1.40) with a CoolSnap HQ2 camera at ambient temperature. Softworx v6.1 was used for acquisition and (Figure legend continues)
between strains (WT versus ΔPf92) and serum concentrations (10 versus 100%). We tested for an interaction effect, and no significant effect modification was identified. One-way ANOVA was used to test whether a difference existed between WT and ΔPf92 in either 10 or 100% serum (Fig. 6C).

**Results**

**Merozoites recruit human complement regulators FH and FHL-1 to their surface**

The AP of complement is activated upon the release of merozoites from infected erythrocytes (37). We determined whether FH and FHL-1, the major soluble human complement regulators of the AP, are recruited to the merozoite surface as a mechanism of complement evasion. Purified merozoites were exposed to NHS, washed to remove adventitious binders, and tightly bound proteins were eluted using nonreducing protein sample buffer. The predicted molecular mass for FH and FHL-1 is 155 and 42 kDa, respectively, and both these proteins migrate on SDS-PAGE in nonreducing conditions at 145 and 35 kDa (Fig. 1A). Using an anti-FH Ab, we concluded that FH and FHL-1 were present in the eluates from merozoite pellets (Fig. 1A). Because C3b, iC3b, and C3d are natural ligands for FH, we tested whether FH recruitment depends on C3 fragment deposition on merozoites. We detected substantial C3 fragment deposition upon NHS exposure (Fig. 1A). Complement inactivation by heat (NHS-HI) reduced C3 deposition with minor residual binding observed, but no smearing of higher molecular bands, which is characteristic of C3 activation to C3b, was observed, whereas inactivation with EDTA (NHS-E) resulted in the loss of C3 binding (Fig. 1A). However, FH was still present in the eluates from merozoite pellets in NHS-HI and NHS-E, suggesting that its recruitment was independent of C3 fragment deposition (Fig. 1A). In contrast, FH-L1 recruitment was sensitive to heat inactivation of NHS, but not to the addition of EDTA to active serum (Fig. 1A, NHS-HI versus NHS and NHS-E). We also observed FH recruitment using 3D7 and W2mef parasites (Fig. 1B).

To visualize whether FH and FHL-1 were recruited to the merozoite surface, we used immunofluorescence staining with anti-FH Ab to detect FH and FHL-1 and an anti-AMA1 Ab to demarcate the surface of the merozoites. In the presence of NHS, NHS-HI, and NHS-E, FH and FHL-1 were colocalized to the periphery of the merozoite (Fig. 1C). As expected, FH staining was absent in buffer control. These results confirm that FH from human serum is recruited to the merozoite surface and that this process does not require prior deposition of C3 fragments (Fig. 1A).

Flow cytometry was used to examine the proportion of merozoites in a population that binds FH. We used a *P. falciparum* strain that expresses GFP, whereas FH binding to cells was detected using anti-FH Ab. The results show that between 40 and 60% of GFP+ merozoites were able to recruit FH in NHS, NHS-HI, and NHS-E conditions, compared with a negligible population in the absence of any serum (Fig. 6C).

**P. falciparum binds to CCP 5–6 of FH and FHL-1**

We used a series of recombinant FH fragments to identify the region of FH that interacts with *P. falciparum* merozoites (Fig. 2A, 2B, Supplemental Fig. 1). Merozoites were incubated with equimolar concentrations of CCP 1–4, CCP 6–8, CCP 8–15, CCP 12–13, CCP 15–18, CCP 15–19, CCP 18–20, and CCP 19–20, as well as positive controls, FH and FHL-1. After washing, tightly bound proteins from the merozoite pellets were analyzed by Western blotting. We observed that only full-length FH and FHL-1 bound to
P. falciparum merozoites (Fig. 2C). Flow cytometry and immunofluorescence confirmed these results except they indicated binding of CCP 6–8, as well as FH and FHL-1 (Fig. 2D and 2E, respectively). Whereas 70% of the merozoite population bound FH and FHL-1, only 20% of the population bound CCP 6–8 (Fig. 2D).

These results suggested that CCPs 5 and 6 are key to the interaction between FH and merozoites. Although we were unsuccessful in expressing a soluble form of CCP 5–6, we generated a fragment containing CCP 4–6 (Supplemental Fig. 2). CCP 4–6 binds merozoites efficiently, whereas CCP 1–4 showed no binding as expected (Fig. 2F, 4–6 versus 1–4 lanes). In addition, CCP 4–6 was effective at blocking the binding of equimolar purified FH to merozoites (Fig. 2F, FH 4–6 versus FH lane).

**MSP P92 recruits FH and FHL-1**

To identify the MSP responsible for recruiting FH, we performed immunoprecipitation assays using anti-FH Ab in the presence or absence of purified FH and solubilized merozoite pellets as a source of lysate followed by mass spectrometry analyses. P92 was identified as a potential candidate and was detectable only in the presence of FH (Supplemental Fig. 3A). To confirm this result, we used the same immunoprecipitation conditions as described earlier and analyzed the eluates by Western blotting using anti-Pf92 Abs. We detected P92 in the +FH lane and not in the −FH lane (Fig. 3A). Additional immunoprecipitation assays were performed using anti-Pf92 and anti-Pf12 Abs in the presence of parasite lysates and purified FH (Fig. 3B). Although both Pf12 and Pf92 could be immunoprecipitated by anti-Pf92 and anti-Pf12, respectively, only P92 was able to immunoprecipitate FH in a complex (Fig. 3B).

We predicted that FHL-1 and CCP 4–6 should also form a complex with P92 and performed immunoprecipitation experiments in which anti-FH Abs were incubated with parasite lysate with the addition of FHL-1, CCP 4–6, or buffer. Both FHL-1 and 4–6 were successfully immunoprecipitated in a complex with P92 (Fig. 3C). As expected, no P92 was detected in the control lacking FH protein fragments (Fig. 3C, −FH lane). We also performed the reciprocal immunoprecipitations using either anti-Pf92 or anti-Pf12 Abs incubated with parasite lysate with either FHL-1 or CCP 4–6 added. Pf92 and Pf12 were successfully immunoprecipitated (Fig. 3D); however, only Pf92 could immunoprecipitate a complex containing either FHL-1 4–6 (Fig. 3D, α-Pf92 IP versus α-Pf12 IP lanes).

The P. falciparum merozoite is coated with ~30 different surface coat proteins. To determine whether Pf92 is the only surface coat protein to interact with FH, we constructed Pf92 knockout transgenic parasites (ΔPf92) and used anti-Pf92 Abs to determine expression patterns (Fig. 4A, Supplemental Fig. 4). Anti-Pf92 Abs did not detect Pf92 in the ∆Pf92 line, whereas there was clear expression in both WT and ∆Pf12 lines (33). WT and ∆Pf92 merozoites were incubated in buffer, NHS, NHS-HI, or NHS-E to examine FH recruitment. Whereas WT merozoites were able to recruit FH and FHL-1 in all conditions, ∆Pf92 merozoites were deficient in their ability to recruit FH and FHL-1 (Fig. 4B). Using anti-Pf12 Abs, we are able to show that we obtained a reliable amount of merozoites in these assays for both WT and ∆Pf92 conditions (Fig. 4B). In addition, ∆Pf92 merozoites showed the absence of Pf92 expression (Fig. 4B). To ensure that deletion of other MSPs does not affect FH binding, we examined ΔMSP3 and ΔMSPDBL1 merozoites and observed that FH was recruited to the same extent as in WT parasite (44) (Fig. 4C). These results show that Pf92 is primarily responsible for recruiting FH to the merozoite.

**P. falciparum–bound FH and FHL-1 remains functionally active**

One mechanism by which FH regulates complement is as a cofactor for Factor I–mediated cleavage of C3b to its less active form iC3b (45). The functional site for cofactor activity is present within CCP 1–4 of FH and FHL-1. In a cofactor activity assay, we observed that purified FH, FHL-1, and CCP 1–4 served as functional cofactors for Factor I cleavage of C3b into iC3b (Fig. 5A). The inactivation of C3b was dependent on the addition of FH fragments because there was no detected cleavage when Factor I was incubated alone with C3b (Fig. 5B). To ascertain whether cofactor activity was retained after recruitment to the merozoite surface, we incubated merozoites with FH, FHL-1, and CCP 1–4, washed

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**FIGURE 4.** ΔPf92 merozoites are unable to recruit FH and FHL-1. Molecular mass marker in kDa is shown on the left-hand side of the blots. (A) Saponin pellets of late-stage schizonts from WT, ΔPf92, and ΔPf12 parasites were denatured in nonreducing sample buffer, and proteins were separated on SDS-PAGE gels before analysis by Western blotting with polyclonal anti-Pf92 Abs. Membranes were subsequently stripped and probed for the related protein Pf12 using anti-Pf12 Abs. (B) WT and ΔPf92 merozoites were deposited with NHS, NHS-HI, NHS-E, or buffer for 10 min at 37°C. After washing, cell pellet proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-FH Ab to detect binding of FH and FHL-1 to merozoites. After stripping, membranes were probed with anti-Pf92. The lighter higher band highlighted with an asterisk (*) represents a cross-reactive protein present in lysates. After this, the membrane was restripped and probed with anti-Pf12 Abs as a loading control. (C) WT, ΔMSP3, and ΔMSPDBL1 merozoites were deposited with NHS for 10 min at 37°C. After washing, cell pellet proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-FH Ab to detect binding of FH to merozoites. After stripping, membranes were probed with anti-AMA1 as a loading control.
Western blotting. (b) Merozoites were incubated with 0.2 μg C3b in the absence (−) or presence (+) of 20 ng Factor I and subsequently incubated at 37°C for 2 h. Supernatants were then run on SDS-PAGE and analyzed by Western blotting. (C) WT merozoites were deposited with 0.5 μM FH, FHL-1, or CCP 1–4 (solution), or merozoites preincubated with CCP 1–4 and after washing were incubated with 0.2 μg C3b in either the absence (−) or presence (+) of 20 ng Factor I at 37°C for 2 h. Supernatants were run on SDS-PAGE and analyzed by Western blotting. (D) Forty nanograms FH, CCP 1–4 (solution), or ΔPf92 merozoites deposited with 0.5 μM C3b, and the C3b cleavage pattern was analyzed by Western blotting. Cofactor activity was detected for merozoites preincubated with FH and FHL-1 (Fig. 5C), but not for merozoites preincubated with CCP 1–4. This shows that FH and FHL-1 are recruited to merozoite surface and still retain cofactor activity. The lack of C3b cleavage in the case of CCP 1–4 (which does not bind merozoites) confirms that the measured cofactor activity is derived from specific merozoite recruitment and not a nonspecific carryover of regulators (Fig. 5C).

We also tested the ability for ΔPf92 merozoites to regulate C3b cleavage on their surfaces. Both FH and CCP 1–4 displayed functional cofactor activity in solution; however, no cofactor activity was observed for ΔPf92 merozoites exposed to either FH or CCP 1–4 (Fig. 5D). It is clear that ΔPf92 merozoites no longer recruit FH to their surfaces and as such cannot mediate cleavage of C3b to its inactive form. Therefore, recruitment of functionally active FH and FHL-1 to WT merozoites provides a mechanism for protection from complement activation.

FH binding protects merozoites from complement-mediated destruction

One direct consequence of complement deposition is pathogen lysis. We wanted to determine whether ΔPf92 parasites, which are unable to recruit FH, would show increased destruction of merozoites upon exposure to serum. Previous work has demonstrated the ability to identify distinct populations of ethidium bromide–stained merozoites by flow cytometry and that loss of nuclear staining can occur under complement duress (2, 42). Therefore, we adapted a similar technique to monitor the percentage of EtBr⁺ merozoites after incubation with NHS compared with NHS-HI. We observed no change in merozoite numbers when WT merozoites were incubated in 10% serum (p = 0.89; Fig. 6A). However, when incubated in 100% serum, a modest 1.8-fold reduction of WT merozoites was observed in NHS compared with NHS-HI (p = 0.377; Fig. 6B). However, incubation in 100% serum led to a 7-fold loss of intact merozoites between NHS and NHS-HI (p = 0.023; Fig. 6A). There was no significant reduction in ΔPf92 merozoites numbers after incubation in 10% NHS (p = 0.377; Fig. 6B). However, incubation in 100% serum led to a 7-fold loss of intact merozoites between NHS and NHS-HI (p = 0.0037; Fig. 6B). The average fold-change of ΔPf92 parasites was significantly elevated compared with that of WT parasites in 100% serum (p = 0.0004; Fig. 6C). These results suggest the recruitment of FH to the merozoite surface via Pf92 leads to a reduction in complement-mediated lysis of merozoites.

We conducted growth assays with WT and ΔPf92 parasites in the presence of NHS or NHS-HI. Although both WT and ΔPf92 showed reduced growth in NHS as compared with NHS-HI, ΔPf92 had significantly reduced growth compared with the WT strain in NHS (p = 0.0081; n = 12; Fig. 6D). We also observed significant reduced growth in ΔPf92 as compared with WT with four cycles of replication in NHS (Supplemental Fig. 4C). Furthermore, we detected substantial C3 fragment deposition with increasing time upon NHS exposure for both WT and ΔPf92 strains (Fig. 6E, top panel). However, using an anti-AMA1 Ab as a parasite loading control, we observed that ΔPf92 showed decreasing levels of

FH-L-1, or CCP 1–4 and after washing were incubated with 0.2 μg C3b in either the absence (−) or presence (+) of 20 ng Factor I at 37°C for 2 h. Supernatants were run on SDS-PAGE and analyzed by Western blotting. (D) Forty nanograms FH, CCP 1–4 (solution), or ΔPf92 merozoites deposited with 0.5 μM solutions of each of these were incubated with 0.2 μg C3b in either the absence (−) or presence (+) of 20 ng Factor I at 37°C for 2 h. Supernatants were run on SDS-PAGE and analyzed by Western blotting.
AMA-1, suggesting the loss of ΔPf92 merozoites upon NHS exposure as compared with WT strain (Fig. 6E, bottom panel). These data support the hypothesis that recruitment of FH protects the malaria parasite from complement-mediated destruction.

**Discussion**

Pathogens that are exposed to serum need strategies to survive the assault of the complement system. The AP launches a very rapid response and can coat a bacterium with $10^8$ copies of C3b in 10 min (46). In this article, we add *P. falciparum* blood-stage merozoites to the list of organisms that recruit the human regulators FH and FHL-1 to prevent complement-mediated lysis. Merozoite surface-bound FH retained cofactor activity for Factor I promoting cleavage of C3b to iC3b. Pf92, an MSP from the 6-cys protein family, mediates FH and FHL-1 binding to the merozoite surface through CCP domains 5–6. ΔPf92 merozoites show a strong reduction in FH and FHL-1 binding and exhibit an increase in complement-mediated destruction, consistent with the failure of the complement evasion strategy.

We identified CCPs 5 and 6 as the region within FH that interacts with the merozoite surface (Fig. 2). Interestingly, gametocytes, the sexual stages of *P. falciparum* parasites, recruit FH using CCPs 5–7 to prevent their destruction when exposed to residual human complement in the mosquito midgut, whereas infected RBCs of late-stage parasites use CCP 5 and 20 (34, 35). Studies of group A streptococci and *Neisseria* also show that CCPs 6 and 7 of FH are exploited for recruitment. Pathogens might bind CCPs 5–7 because this region is also present in FHL-1, therefore allowing pathogens to recruit both complement regulators (47). CCP 19–20 is also a recurring target site among pathogens (47). Intriguingly,
this binding site may enhance FH function. We found no evidence of CCP 19–20 binding to the merozoite surface. However, ΔPf92 displayed minor residual FH, but not FHL-1, binding in the NHS condition (Fig. 4). Because the NHS condition is the only one with C3b deposited on the merozoite surface, we propose this minor binding may be the result of FH binding to its natural ligand in our assay, rather than specific recruitment by the merozoite. However, we cannot preclude that another MSP recruits FH to the surface via interaction with another region of FH. Despite this, Pf92 is the major FH recruiting protein on merozoites.

An emerging theme is the role of 6-cys family members in immune modulation by malaria parasites. Our study identified Pf92 as the MSP that interacts with FH and FHL-1 (Fig. 3). Pf92 is an abundant protein on the merozoite surface accounting for ~5% of the GPI-anchored MSPs (30). Another 6-cys protein, PfS47, which is expressed on the female gametocyte surface, has been shown to protect the sexual stages of P. falciparum from complement-mediated destruction (Fig. 6). Protection appears to be achieved by interfering with nitration reactions that are a critical component for activation of the system (26). Abs raised to PfS230, another 6-cys protein expressed on the female gametocyte surface, also led to transmission blocking activity when mosquitoes were fed parasitized with Abs in complement active sera, suggesting that the inhibition of PfS230 is leading to destruction by complement (25, 48, 49). An exciting area for future investigations will be the role of 6-cys family of proteins and how they regulate the human immune response.

Rupture of the infected RBCs not only releases merozoites but also a parasite organelle called the digestive vacuole. Digestive vacuoles activate complement and promote complement deposition on bystander cells, which could include merozoites and neighboring RBCs (37, 38). We propose merozoites recruit FH to protect themselves from this additional threat of complement assault. We were able to show that merozoite-bound FH retains cofactor activity and leads to C3b inactivation (to iC3b), providing a molecular mechanism for protection (Fig. 5). This efficient inactivation was not observed with ΔPf92 merozoites, which are unable to recruit FH and as such undergo more significant complement-mediated destruction (Figs. 5, 6). However, the ΔPf92 strain shows only a modest decrease in growth compared with WT parasites, potentially suggesting that other mechanisms may also be providing protection from complement-mediated destruction (Fig. 6).

Apart from interacting with FH and FHL-1, P. falciparum merozoites are known to interact with another complement regulator called Complement Receptor 1 (CR1) that is located on RBC surfaces (50, 51). P. falciparum uses CR1 as an entry receptor into RBCs through binding to a parasite adhesin called PfRh4 (51, 52). It has been suggested that merozoites may play a dangerous game of allowing C3 fragment deposition to occur to more readily allow PfRh4 engagement with the CR1 molecules dispersed across the RBC surface (52). Although iC3b cannot participate in the generation of MAC, it is still a ligand for CR1 (5), and the aforementioned ability of merozoite-recruited FH and FHL-1 to convert C3b to iC3b would remove some of the danger associated with such an approach.

In this study, we found P. falciparum blood-stage merozoites actively recruit the host regulator FH and FHL-1 to their surface. These results offer a new insight into the complex interaction between host and pathogen. However, FH only regulates the AP of complement yet still exerts some protective effect. We predict that merozoites will recruit other host regulators of complement or use other strategies to control activation via the CP.

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Disclosures

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References


