Sexual Stage Adhesion Proteins Form Multi-protein Complexes in the Malaria Parasite *Plasmodium falciparum*

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The sexual phase of the malaria parasite *Plasmodium falciparum* is accompanied by the coordinated expression of stage-specific adhesive proteins. Among these are six secreted proteins with multiple adhesion domains, termed *P. falciparum* LCCL domain-containing protein (*PfCCp*) proteins, which are expressed in the parasitophorous vacuole of the differentiating gametocytes and which are later associated with macrogametocytes. Although the majority of the *PfCCp* proteins are implicated in parasite development in the mosquito vector, their functions remain unknown. In the present study we investigated the molecular interactions between the *PfCCp* proteins during gametocyte development and emergence. Using five different gene-disruptant parasite lines, we show that the lack of one *PfCCp* protein leads to the loss of other *PfCCp* family members. Co-immunoprecipitation assays on gametocyte lysates revealed formation of complexes involving all *PfCCp* proteins, and affinity chromatography co-elution binding assays with recombinant *PfCCp* domains further indicated direct binding between distinct adhesion domains. *PfCCp*-coated latex beads bind to newly formed macrogametes but not to gametocytes or older macrogametocytes 6 or 24 h post-activation. In view of these data, we propose that the *PfCCp* proteins form multi-protein complexes that are exposed during gametogenesis, thereby mediating cell contacts of macrogametes.

The sexual phase of the malaria pathogen *Plasmodium falciparum* begins with the differentiation of predetermined blood stage parasites to gametocytes in the human host and continues with the formation of male and female gametocytes in the midgut of the blood-fed female mosquito vector. Subsequently, the motile male microgamete fertilizes the amotile female macrogamete, and within a day after the blood meal, the resulting zygote transforms into an infective ookinete. Gametocytogenesis and gamete formation are accompanied by the coordinated expression of numerous sexual stage proteins (1, 2), including the epidermal growth factor domain-containing proteins *Pf*s25 and *Pf*s28, as well as the cysteine motif-rich proteins *Pf*s230 and *Pf*s48/45, all of which are considered promising candidates for components of a transmission blocking vaccine (2, 3).

Among the sexual stage proteins that have been recently identified in *P. falciparum* are a highly conserved family of six secreted proteins that are comprised of multiple predicted adhesive domains (4–8). Five of the proteins possess a common *Limulus* coagulation factor C (LCCL) domain and are termed *PfCCp1* through *PfCCp5*, whereas a sixth protein, *PfFNPA*, lacks this domain but shares architectural features that indicate its vertical relationship with *PfCCp5* and warrant its inclusion as a member of the protein family. The *PfCCp* proteins have orthologs in other apicomplexan parasites, including *Toxoplasma* and *Cryptosporidium*, and are known as *PbLAP* proteins in the rodent malaria parasite *Plasmodium berghei* (7–9).

The six *PfCCp* proteins are expressed during gametocytogenesis of *P. falciparum* and localize within the gametocyte parasitophorous vacuole (7, 10, 11). During gamete emergence they are exposed associated to the surface of macrogametes, but expression ceases within a day (7, 11). Gene disruptions of *PfCCp2* or *PfCCp3* resulted in a blocked transition of sporozoites from the mosquito midgut to the salivary glands, showing that these proteins are essential for the parasite development in the vector (7). Gene disruption of *PfCCp4*, on the other hand, did not reveal any knock-out phenotype in the mosquito stages (11). Antibodies directed against select *PfCCp* adhesion domains induce a complement-mediated reduction of exflagellation, making the proteins potential candidates as subunits of transmission blocking vaccines (11).

We have recently reported that *PfCCp1*, *PfCCp2*, and *PfCCp3* co-localize within the parasitophorous vacuole of mature gametocytes (10). The three proteins are co-dependently expressed at the protein but not transcript level, leading to the assumption that they might interact during expression in gametocytes and that the loss of one protein results in degradation of the other two (10). For this study, we generated novel *PfCCp1* and *PfFNPA* gene-disruptant parasites and show that in gene-disruptant gametocytes lacking *PfCCp1*, *PfCCp2*, *PfCCp3*, *PfCCp4*, or *PfFNPA*, the *PfCCp* proteins were either absent or expressed at a decreased protein level. We further...
demonstrate that the six proteins undergo intermolecular interactions and are involved in cell binding of the newly emerged macrogametes. In conclusion, we propose that the PfCCp proteins form multi-protein complexes that are exposed during gamete formation and that might mediate cell contacts of macrogametes.

**EXPERIMENTAL PROCEDURES**

**Gene Identifications**—The following gene identifiers are assigned to the proteins investigated in this study: Pf39, PF11_0098; PfCCp1, PF14_0723; PfCCp2, PF14_0532; PfCCp3, PF14_0067; PfCCp4, PF10185w; PfCCp5, PFA0445w; PfFNPA, PF14_0491; and PfBS, PF10_0205.

**Oligonucleotides**—PfCCp1 5’ SpeI, 5’-ACTAGTTACATG-TCTGAAAGCTTCC; PfCCp1 5’ BglII, 5’-AGATCTTTACTGG-TATTACGTACATTACAG; PfCCp1 3’ Clal, 5’-ATCGATGCAACACATATCTGAATATC TT; PfCCp1 3’ Ncol, 5’-CCATG-GTTAGATGTTGGTGCTC; PfFNPA BamHI, 5’-ATGGAATTCGGGGGTACATGATTATAT; PfFNPA 5’ NotI, TAGCGCCGCCTTAGTATGACCATGGATT; PfCCp1 3’ Ncol, 5’-CCATG-GTTAGATGTTGGTGCTC; PfFNPA BamHI, 5’-ATGGAATTCGGGGGTACATGATTATAT; PfFNPA NotI, TAGCGCCGCCTTAGTATGACCATGGATT; and PfFNPA 3’ NotI, ACATGATGATGCCAGGAG.

**Antibodies**—The following antibodies were used in this study: mouse antiserum against PfCCp1-CC, PfCCp2-Disc, have integrated plasmid by double cross-over homologous recombination were selected with pyrimethamine and ganciclovir (15). A pyrimethamine-resistant polyclonal line was established, and clones were isolated by limiting dilution. Correct integration of the targeting plasmid into the genomic locus was verified by PCR and Southern blot analyses diagnostic of a disrupted locus. PCR was performed with 100 ng of genomic DNA from wild type or disruptant parasites for 35 cycles of amplification and with an annealing temperature of 50 °C, using the above mentioned primers a, b, b’, c’, and d (shown in supplemental Fig. S1). Southern blotting was performed with 10 μg of genomic DNA from wild type or disruptant parasites, digested with KpnI plus BamHI, separated by 0.8% agarose gel electrophoresis, and transferred to Hybond N+ (Amersham Biosciences). A 1.1-kb hybridization probe corresponding to the 5’-coding region of PfCCp1 was amplified with the primers PfCCp1 5’ SpeI and PfCCp1 5’ BglII. The probe was labeled with digoxigenin, and hybridization was performed at 48 °C using a DIG High Prime DNA labeling and detection starter kit II (Roche Applied Science), according to the manufacturer’s instructions. Lack of PfCCp1 transcripts in the disruptant clone was confirmed by RT-PCR. RNA from mature wild type and disruptant gametocytes was isolated with TRIzol (Invitrogen) according to the manufacturer’s instructions, treated with DNase I (Invitrogen), and purified on RNeasy MinElute Cleanup columns (Qiagen). RNA was reverse-transcribed using Superscript II that was primed with random hexanucleotides (Invitrogen). PCR was performed for 35 cycles of amplification using the oligonucleotides e and f. The absence of genomic DNA contamination was confirmed by PCR amplification on sham-treated RNA samples that lacked reverse transcriptase. Absence of protein expression was assessed by Western blot and indirect immunofluorescence assay, as described below. Furthermore, PfFNPA gene-disruptant parasites were generated by single homologous recombination using the vector pCAM-BS2 (17, 18). A 568-bp fragment of the PfFNPA gene was amplified by PCR from P. falciparum NF54 genomic DNA using the primer pair PfFNPA BamHI and PfFNPA NotI, which included a stop codon. The PCR product was cloned into the restriction site BamHI/NotI of the pCAM-BS2 vector. Further steps were carried out as described above, except that selection of stable transfected parasites, which had integrated plasmid by single cross-over homologous recombination, was pursued using blasticidin. PCR with genomic DNA from wild type or disruptant parasites was performed for 33 cycles of amplification and with annealing temperatures ranging from 42 to 50 °C using the primers s, t, u, and v (shown in supplemental Fig. S2). Southern blotting was carried out with 2 μg of wild type parasites or parasites of the PfFNPA-KO (knock-out) clone 1H4, digested with MfeI and NcoI. A hybridization probe of 825 bp corresponding to the 5’-coding region of PfFNPA was amplified with the primers w and x. Southern blotting was performed above, using a hybridization temperature of 35 °C. RNA isolation and transcript level analysis was conducted as below. For RT-PCR oligonucleotides q and r were used as well as Pf39-specific oligonucleotides y and z as a control.

**Parasite Culture**—Mature gametocytes of the P. falciparum NF54 isolate were cultivated in vitro as described (12). Gametocyte activation and gametogenesis was induced by incubating mature gametocyte cultures in 100 μM xanthurenic acid/PBS, pH 8.5 (13, 14) or human serum for 15 min at room temperature.

**Generation of Gene-disruptant Parasites**—PfCCp1 gene-disruptant parasites were generated by double homologous recombination using the targeting vector pHHT-TK (ATCC, Manassas, VA) (15). Two fragments of the PfCCp1 gene corresponding to the 5’- and 3’-coding regions of 1,138 and 1,522 bp, respectively, were amplified by PCR from P. falciparum NF54 genomic DNA using the above mentioned primer pairs PfCCp1 5’ SpeI and PfCCp1 5’ BglII as well as PfCCp1 3’ Clal and PfCCp1 3’ Ncol. These PCR products were sequentially cloned into the restriction sites SpeI/BglIII and Clal/Ncol of the pHHT-TK vector. The gene targeting disruption plasmid was introduced into the P. falciparum isolate NF54 via the erythrocyte-loading method (16), and stable transfected parasites that
and PfCCp3-SR (7); PfCCp1rp1, PfCCp1rp5, PfCCp2rp3, PfCCp3rp3, PfCCp4rp1, PfCCp4rp3, PfCCp5rp1, PfCCp5rp2, PfFNPArp1, and PfFNPArp2 (11); mouse antisera against peptide PfCCp4rp2 (11); mouse antisera against the endoplasmic reticulum-associated protein Pf39 (19); and goat antisera against GST tag (Amersham Biosciences) or mouse antisera against His6 tag (GE Healthcare).

RNA Isolation and Transcript Level Analysis—Gametocytes were enriched by Percoll gradient purification (20), and RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA preparations were treated with RNase-free DNase I (Invitrogen) to remove genomic DNA contamination, followed by phenol/chloroform extraction and ethanol precipitation. Synthesis of cDNA was accomplished using a SuperScript II cDNA synthesis kit (Invitrogen). RT-PCR assays were performed with 125 ng of cDNA using gene specific primers by conventional thermocycling (25 cycles) separated by 2% agarose gel electrophoresis. The following primers were used: PfCCp1, g and h; PfCCp2, i and j; PfCCp3, k and l; PfCCp4, m and n; PfCCp5, o and p; and PfFNPNA, q and r.

Western Blot Analysis—Pellets of enriched gametocytes were resuspended in PBS and SDS-PAGE loading buffer as described (11). Parasite proteins were separated by SDS-PAGE electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) according to the manufacturer’s instructions. The membranes were blocked for nonspecific binding by incubation in Tris-buffered saline containing 5% skim milk and 1% bovine serum albumin fraction V, followed by immune recognition for 2 h at room temperature with mouse immune sera specific to PfCCp proteins, Pf39, or to GST- and His6/SUMO fusion protein partner control. After washing, the membranes were incubated for 1 h at room temperature with an alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich) and developed in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Sigma-Aldrich) for 5–30 min. Scanned blots were processed using Adobe Photoshop CS software.

Indirect Immunofluorescence Assay—Activated or nonactivated gametocyte cultures were air dried on slides and fixed for 10 min in −80 °C methanol. For membrane permeabilization and blocking of nonspecific binding, the fixed cells were incubated in 0.01% saponin, 0.5% bovine serum albumin, and 1% neutral goat serum (Sigma-Aldrich) in PBS twice for 30 min each. The preparations were then incubated for 1.5 h at 37 °C with anti-PfCCp mouse immune sera. Binding of primary antibody was visualized using fluorescence-conjugated goat anti-mouse secondary antibodies (Alexa Fluor 488 and 594; Molecular Probes). Counterstaining was performed using 0.05% Evans Blue in PBS for 1 min (Sigma-Aldrich). Labeled specimens were examined by confocal laser scanning microscopy using a Zeiss LSM 510. The digital images were processed using Adobe Photoshop CS software.

Co-immunoprecipitation Assay—Enriched gametocytes were lysed in 0.5% saponin in PBS, homogenized, and sonicated for 1 min. The homogenate was centrifuged at 16,000 × g, and prepreparation of the supernatant was performed by consecutive incubation with 5% (v/v) preimmune mouse sera and 20 μl of protein G–beads (Santa Cruz Biotechnology) for half an hour each at 4 °C. After centrifugation at 3,400 × g, the supernatant was then incubated for 1 h at 4 °C with 5% (v/v) polyclonal mouse antisera against PfCCp proteins or with antisera against Pf39 as a negative control. A volume of 20 μl of protein G beads was added and incubated for another hour. The beads were then centrifuged, washed five times with PBS, mixed with an equal volume of loading buffer, and loaded onto a 12% SDS gel. Precipitated proteins were then detected via Western blot analysis as described above. To investigate the ability of precipitated PfCCp proteins to bind to recombinant PfCCp adhesion domains, co-immunoprecipitation was performed as described using anti-PfCCp1 antibodies, and the precipitated complex was then incubated with purified recombinant PfCCp3rp3-GST or GST alone, both of which were prepurified with beads as described above, overnight at 4 °C (for expression and affinity purification; see below). The samples were washed five times with PBS and loaded onto 12% or 15% SDS gels. Precipitated proteins were detected via Western blot analysis as described above.

Affinity Chromatography Co-elution Binding Assay—Recombinant proteins were expressed as fusion proteins with either a GST tag using the pGEX-4T1 vector (Amersham Biosciences) or a His6/SUMO tag using the pSUMO vector as described (see Fig. 3B for domain specificity) (11). Recombinant proteins PfCCp1rp1, PfCCp1rp6, PfCCp3rp1, PfCCp3rp3, PfCCp3rp4, PfCCp4rp4, and Pf39 were tagged with GST and used as bait, whereas the recombinant proteins PfCCp1rp1, PfCCp1rp2, PfCCp1rp3, PfCCp1rp5, PfCCp2rp3, PfCCp2rp4, PfCCp3rp1, PfCCp3rp3, PfCCp5rp1, PfCCp5rp2, and PfFNPArp2 were expressed with a His6/SUMO tag and functioned as prey in the affinity chromatography co-elution binding experiments. The proteins were expressed in BL21 (DE3) RIL cells according to the manufacturer’s protocol (Stratagene). The GST fusion protein expressing bacteria cells were lysed three times using a French press and 2 min via sonication. The lysed cells were centrifuged at 30,000 × g, and the supernatant was mixed with GST-Sepharose (Amersham Biosciences) and applied to a Poly-Prep chromatography column (Bio-Rad) at 4 °C. Supernatants containing His6- or SUMO-tagged potential interaction partners were loaded onto the GST fusion protein-bound column. The column was washed five times with PBS. The last washing step was investigated via Western blot analysis for the absence of GST-tagged and His6/SUMO-tagged proteins. The mutual binding abilities were investigated via co-elution, using a 50 mM Tris/HCl elution buffer, pH 8, containing 10 mM reduced glutathione. Via SDS-PAGE electrophoretic separation of eluates, the interaction partners were assayed by Western blot analysis using anti-His6 antibodies (GE Healthcare) and anti-GST antibodies (Amersham Biosciences). Co-elutions of recombinant GST tag protein with His6/SUMO tag protein or of Pf39 protein with PfCCp1rp1 protein were used as negative controls. For binding studies on endogenous PfCCp proteins, GST-tagged PfCCp3rp3 bait protein was applied to the columns. Gametocyte lysate was prepared as described for the co-immunoprecipitation assay, diluted with PBS, and loaded to the bait-bound column. Co-elution binding assays were performed as described above. The elutions were concentrated with Amicon ultra filter devices (Millipore) and analyzed for
the presence of \textit{PfCCp}3rp3-GST and endogenous \textit{PfCCp}1 proteins by Western blotting. Blotting with antibodies against \textit{Pfs}39 was used as negative control.

**Latex Bead-mediated Cell Binding Assay**—For activation, 5x10^2 carboxylate-modified, yellow-green fluorescent polystyrene latex beads with a diameter of 1 µm (Sigma-Aldrich) were incubated in 50 µl of 500 mM MES (2-(N-morpholino)-ethanesulfonic acid; Roth) pH 5–6, 40 µl of 100 mg/ml N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma-Aldrich), and 410 µl of H_2O_{bused} for 2 h at room temperature under rotation. After washing twice with 50 mM MES, the beads were conjugated with 5 µg of the recombinant proteins \textit{PfCCp}3rp1 or \textit{PfCCp}3rp3 (GST-tagged), \textit{PfCCp}1rp1 or \textit{PfCCp}2rp3 (His$_x$/SUMO-tagged), or, as negative controls, with the GST-portion alone or the His$_x$ fusion protein 5-helix (21) in 50 mM MES for 2 h at room temperature under rotation. Expression and purification of the recombinant proteins was conducted as described (see Fig. 3B for domain specificity) (11).

To remove unbound proteins and block unspecific binding sites, the coated latex beads were washed twice with PBS and incubated overnight in 1% bovine serum albumin, 0.05% Tween 20 in PBS, pH 7.4, at 4 °C, followed by washing three times in PBS. The coated beads were mixed with either nonactivated gametocyte cultures or gametocyte cultures 15 min, 6 h, or 24 h post-activation or with \textit{PfCCp}3-KO gametocytes 15 min post-activation, which were fixed in 4% paraformaldehyde overnight and washed three times in PBS. After incubation overnight at 4 °C, the number of macrogametes (15 min, 6 h, and 24 h post-activation), nonactivated gametocytes and erythrocytes that bound the \textit{PfCCp}-coated latex beads were counted for 20 optical fields (400 x magnification) using an Zeiss Axioshot microscope. This corresponds to a total number of ~100 gametocytes or gametocytes and of ~1000 erythrocytes. In total, two to four independent assays were performed, and the mean values were calculated. The respective cell types were distinguished because of their morphological appearance; mature (stage V) gametocytes were crescent-shaped, and macrogametocytes were round, contained multiple hemozoin particles, and were smaller and more translucent than erythrocytes. Binding of latex beads to macrogametocytes was subsequently verified by immunofluorescence assay using antibodies against \textit{Pfs}25 in combination with \textit{Alexa Fluor} 596-conjugated secondary antibodies.

**RESULTS**

**Generation of Gene-disruptant Parasites**—The \textit{PfCCp}1 and \textit{PfFNPA} gene loci were disrupted to complete the panel of targeted gene knock-outs of \textit{PfCCp} family members. The \textit{PfCCp}1 targeting disruption plasmids were introduced into the \textit{P. falciparum} isolate NF54, and stable transfected parasites were selected with pyrimethamine and ganciclovir to drive plasmid integration by double cross-over homologous recombination (supplemental Fig. S1) (15). A pyrimethamine-resistant polyclonal line was established, and clonal lines were isolated by limiting dilution. Correct disruption of \textit{PfCCp}1 was verified by diagnostic PCR (supplemental Fig. S1). Southern blot analysis of Kpnl- and BamH1-digested genomic DNA from wild type and knock-out parasites showed correct disruption of the \textit{PfCCp}1 locus and confirmed that double cross-over occurred (supplemental Fig. S1). \textit{PfCCp}1 transcripts were not detected by RT-PCR in the gametocyte stages of disruptant parasites (supplemental Fig. S1), and the lack of \textit{PfCCp}1 expression was confirmed by Western blot analysis of gametocyte protein extracts isolated from \textit{PfCCp}1-KO parasites compared with a wild type control (Fig. 1A) and by immunofluorescence assay (supplemental Fig. S1). \textit{PfCCp}1 gene-disruptant parasites were capable of propagation of asexual intraerythrocytic stages and production of gametocytes comparable with wild type, indicating that \textit{PfCCp}1 is not essential for intraerythrocytic development. Moreover, gamete emergence and exflagellation of male gametes appeared normal in \textit{in vitro} emergence assays.

Furthermore, we were able to disrupt the locus of \textit{PfFNPA} by means of a pCAM-BSD targeting disruption plasmid, enabling the selection of stable transfectants with blasticidin, which lead to plasmid integration by single cross-over homologous recombination (supplemental Fig. S2). Disruption of the \textit{PfFNPA} gene locus was verified for a clonal line via diagnostic PCR and Southern blot (supplemental Fig. S2). Absence of \textit{PfFNPA} protein was shown by Western blot analysis of gametocyte protein extracts (Fig. 1A), as well as by immunofluorescence assay (supplemental Fig. S2), compared with protein expression in wild type gametocytes, respectively. \textit{PfFNPA} gene-disruptant parasites were capable of propagation of asexual intraerythrocytic stages and production of gametocytes comparable with wild type, and gamete emergence and exflagellation appeared normal in \textit{in vitro} emergence assays.

**Co-dependent Expression of PfCCp Proteins**—We have previously shown that protein but not transcript expression of \textit{PfCCp}1 and \textit{PfCCp}2 is abolished in \textit{PfCCp}3 gene-disruptant lines and that \textit{PfCCp}1 and \textit{PfCCp}3 protein expression decreases in \textit{PfCCp}2-KO clones (10). To determine whether co-dependent protein expression extends to other \textit{PfCCp} proteins, we investigated gene-disruptant lines of the newly generated \textit{PfCCp}1-KO and \textit{PfFNPA} protein (described above, as well as of \textit{PfCCp}2-KO and \textit{PfCCp}3-KO (7), and of \textit{PfCCp}4-KO (11). Western blot analysis revealed that expression of \textit{PfCCp}1, \textit{PfCCp}2, \textit{PfCCp}3, \textit{PfCCp}5, and \textit{PfFNPA} was abolished in the \textit{PfCCp}1-KO, \textit{PfCCp}2-KO, and \textit{PfCCp}3-KO lines, whereas the respective \textit{PfCCp} proteins were present at a decreased expression level in the \textit{PfCCp}4-KO and the \textit{PfFNPA} (Fig. 1A). Screening with antibodies against the endoplasmic reticulum-associated protein, \textit{Pfs}39, was used as a control for equal loading. Because of the low efficiency of the \textit{PfCCp}4 antibody in Western blot analysis, co-dependent expression for this protein was investigated by immunofluorescence assay and revealed that \textit{PfCCp}4 remains homogeneously expressed associated to the surface of \textit{PfCCp}1-KO, \textit{PfCCp}2-KO, \textit{PfCCp}3-KO, and \textit{PfFNPA}-KO gametocytes (Fig. 1B). Immunofluorescence assays further revealed that all \textit{PfCCp} proteins are present on the surface of \textit{PfCCp}4-KO gametocytes (Fig. 1C), indicating that none of the five proteins are targeted to other compartments.

To confirm that the lack of \textit{PfCCp} expression manifests at the protein but not the transcript level, mRNA expression was investigated in the respective gene-disruptant gametocytes using RT-PCR. All of the \textit{PfCCp} transcripts were present in the \textit{PfCCp}1-KO, \textit{PfCCp}2-KO, and \textit{PfCCp}3-KO parasites (supple-
no transcript expression was observed for \( \text{PfCCp1} \). Our data indicate that the six \( \text{PfCCp} \) family members are co-dependently expressed in gametocytes on the protein but not transcript level and that the lack of one of these proteins leads to either partial or to complete loss of the \( \text{PfCCp} \) proteins.

Molecular Interactions between \( \text{PfCCp} \) Proteins—Co-dependent expression of the \( \text{PfCCp} \) proteins suggested protein-protein interactions, in agreement with the previously described co-localization in the parasitophorous vacuole (7, 10, 11). To investigate possible molecular interactions, we initially performed co-immunoprecipitation assays on gametocyte lysates, as described (11). Prominent protein bands for \( \text{PfCCp1}, \text{PfCCp2}, \text{PfCCp3}, \text{PfCCp5}, \text{and PfFNPA} \) were detected by Western blot analysis of gametocyte lysates, when these were individually precipitated with antibodies against either one of these proteins (Fig. 2). Because of the above mentioned low efficiency of the \( \text{PfCCp4} \) antibody in Western blot analysis, interactions of \( \text{PfCCp4} \) with other \( \text{PfCCp} \) proteins were detected by precipitating with the respective \( \text{PfCCp4} \) antibody and showed binding of the protein to \( \text{PfCCp1}, \text{PfCCp2}, \text{PfCCp3}, \text{and PfFNPA} \) (Fig. 2). The precipitated proteins migrated at the expected molecular masses of 185 kDa (\( \text{PfCCp1}, \text{PfCCp2} \)), 150 kDa (\( \text{PfCCp3} \)), 125 kDa (\( \text{PfCCp5} \)), and 100 kDa (\( \text{PfFNPA} \)). For \( \text{PfCCp2} \) and \( \text{PfCCp4} \), second protein bands with molecular masses of ~70 and 60 kDa were detected, respectively, as has been described (11). For \( \text{PfCCp3} \), in some occasions a second protein band, running at a molecular mass of ~90 kDa, was detected, which might be due to processing of the protein. No \( \text{PfCCp} \) bands were detectable when lysates were precipitated with antibodies against \( \text{PfFNPA} \) (Fig. 2). An additional negative control was performed by screening with antibodies against \( \text{PfBS} \) after precipitation with each of the six \( \text{PfCCp} \) antibodies, and no protein bands were detected (Fig. 2). Furthermore, anti-\( \text{PfCCp1} \) mouse serum was used for precipitation in PBS instead of gametocyte lysate, and the precipitate was screened by Western blot analysis with goat anti-mouse sec-

**FIGURE 1. Co-dependent expression of \( \text{PfCCp} \) proteins.** A, Western blot analysis of gametocyte lysates from \( \text{PfCCp1-KO} \), \( \text{PfCCp2-KO} \), \( \text{PfCCp3-KO} \), \( \text{PfCCp4-KO} \), and \( \text{PfFNPA-KO} \) parasite lines using \( \text{PfCCp} \)-specific mouse antisera. \( \text{PfCCp1}, \text{PfCCp2}, \text{PfCCp3}, \text{PfCCp5}, \text{and PfFNPA} \) proteins are absent in the \( \text{PfCCp1-KO} \), \( \text{PfCCp2-KO} \), and \( \text{PfCCp3-KO} \) lines and expressed at a decreased protein level in \( \text{PfCCp4-KO} \) and \( \text{PfFNPA-KO} \) parasites. Protein bands migrate at the expected molecular masses of 185 kDa (\( \text{PfCCp1}, \text{PfCCp2} \)), 150 kDa (\( \text{PfCCp3} \)), 125 kDa (\( \text{PfCCp5} \)), and 100 kDa (\( \text{PfFNPA} \)), as indicated by arrowheads. An additional protein band at a molecular mass of 70 kDa was detectable for \( \text{PfCCp2} \). Antibodies against the endoplasmic reticulum-associated protein \( \text{PfBS} \) (39 kDa) were used as loading control. B, indirect immunofluorescence assay using \( \text{PfCCp4} \) mouse antiserum demonstrated that \( \text{PfCCp4} \) is expressed in \( \text{PfCCp1-KO} \), \( \text{PfCCp2-KO} \), \( \text{PfCCp3-KO} \), and \( \text{PfFNPA-KO} \) gametocytes (green). Erythrocytes were counterstained with Evans Blue (red). C, indirect immunofluorescence assay using \( \text{PfCCp} \)-specific mouse antiserum demonstrated that the proteins \( \text{PfCCp1} \) through \( \text{PfFNPA} \), with the exception of \( \text{PfCCp4} \), are expressed in wild type as well as \( \text{PfCCp4-KO} \) gametocytes. \( \text{PfCCp4} \) is not expressed in \( \text{PfCCp4-KO} \) gametocytes. 1KO, \( \text{PfCCp1-KO} \); 2KO, \( \text{PfCCp2-KO} \); 3KO, \( \text{PfCCp3-KO} \); 4KO, \( \text{PfCCp4-KO} \); FK0, \( \text{PfFNPA-KO} \); WT, wild type. Bar, 5 μm.
Complex Formation of PfCCp Proteins

**FIGURE 2. Multi-protein complex formation of the six PfCCp proteins in gametocytes.** Interactions between PfCCp proteins were determined by co-immunoprecipitation assays with wild type gametocyte lysates precipitated with mouse antibodies against PfCCp1 through PfFNPA (indicated above the horizontal line of each blot), followed by Western blot analysis using the same antibodies individually (indicated above each lane). The protein bands migrate at the expected molecular masses of 185 kDa (PfCCp1, PfCCp2), 150 kDa (PfCCp3), 180 kDa (PfCCp4), 125 kDa (PfCCp5), and 100 kDa (PfFNPA). The additional protein bands at 70, 90, and 60 kDa were detected for PfCCp2, PfCCp3, and PfCCp4, respectively. Antibodies directed against the endoplasmic reticulum-specific protein P39 (39 kDa) were used as a negative control and revealed no binding. Also, no PfCCp protein bands were detected in gametocyte lysates precipitated with antibodies against P39. As a control, mouse serum (MS) was used for precipitation, and the precipitate was blotted with PBS and the alkaline phosphatase-conjugated goat anti-mouse secondary antibody to visualize precipitating antibody, resulting in a smeared protein band at ~55 kDa.

To investigate the molecular interactions between the PfCCp proteins in more detail, affinity chromatography co-elution binding assays were performed. Recombinant proteins corresponding to distinct adhesion domains were expressed for each of the six proteins (for domain regions, see Fig. 3B). Recombinant PfCCp3 adhesion domains, fused to a GST tag, were immobilized to Sepharose and used as baits. Other recombinant PfCCp adhesion domains, fused to a His<sub>S</sub>/SUMO tag, functioned as preys and were incubated with the Sepharose-bound PfCCp3 domains. Bound proteins were eluted after several washing steps. The eluted protein complexes were screened by Western blot analysis, in which the GST-tagged and His<sub>S</sub>/SUMO-tagged proteins were detected via antibodies directed against the respective tags. In an additional set of experiments, GST-tagged PfCCp1rp1 was used as a bait protein and investigated for its interaction with His<sub>S</sub>/SUMO-tagged PfCCp5 and PfFNPA recombinant adhesion domains. Fig. 3A shows a Western blot assay representative of a positive interaction between the recombinant proteins PfCCp3rp3-GST and PfCCp1rp2-His<sub>S</sub>/SUMO and also gives an example for a co-elution binding assay between two proteins (PfCCp3rp4-GST and PfCCp2rp3-His<sub>S</sub>/SUMO) that did not interact. During each interaction experiment, a sample of the last washing step was investigated for the presence of prey or bait proteins and shown to be negative (data not shown). In some cases, lower molecular mass protein bands of GST fusion proteins were detected in addition to the expected full size molecular mass recombinant protein. These protein bands are likely due to contamination of the recombinant protein with truncated recombinant products. In a set of negative controls, GST tag alone was immobilized to Sepharose, and the His<sub>S</sub>/SUMO tag alone was used as prey. In another negative control, secondary antibody only to visualize precipitating antibody. The Western blot resulted in a smeared protein band near 55 kDa, which was also observed in all other lanes (Fig. 2). The results obtained from co-immunoprecipitation assays reveal multiple interactions between the six PfCCp proteins and point to the formation of a multi-protein complex in gametocytes.

In total we investigated interactions between 33 combinations of recombinant PfCCp proteins. Of these pairs, 18 showed adhesive interaction, whereas 15 recombinant protein pairs did not interact in affinity chromatography co-elution binding assays (Fig. 3B). Adhesion domains that were predominant in protein binding interactions include the LCCL domains of all PfCCp proteins and the scavenger receptor (SR) domains of PfCCp3. The two domains were involved in 32% (LCCL) and 19% (SR) of all binding events. Moreover, the apical A, neuropilin, and discoidin domains of PfCCp1 and PfCCp2 might be involved in these interactions.
We additionally examined the ability of recombinant PfCCp domains to bind endogenous PfCCp. In a first set of experiments, affinity chromatography co-elution binding assays were performed using GST-tagged PfCCp3rp3 as bait protein, which was incubated with gametocyte lysate. Western blot analysis on co-eluted samples using anti-PfCCp1 sera revealed binding between recombinant PfCCp3rp3 and endogenous PfCCp1 (Fig. 3C). For control, the samples were blotted with antibodies against Pf39, and the respective blot was negative (data not shown). In a vice versa approach we examined the capability of the recombinant PfCCp3rp3 to bind to the endogenous multi-protein complex. The protein complex, which was precipitated from gametocyte lysate via co-immunoprecipitation using anti-PfCCp1 antibodies, was used as bait and incubated with purified PfCCp3rp3-GST. A prominent PfCCp3rp3 protein band was detected by Western blot analysis using anti-GST antibodies (Fig. 3D). As negative control, the multi-protein complex was incubated with GST alone, and no interactions were detected (Fig. 3D).

Takken together, we demonstrated binding between endogenous PfCCp proteins by co-immunoprecipitation assays, and by co-elution binding assays we further showed binding between recombinant PfCCp proteins and of recombinant PfCCp to endogenous PfCCp protein. In light of our interaction data we conclude that the six PfCCp proteins form multi-protein complexes in the gametocyte parasitophorous vacuole by direct binding through distinct adhesion domains.

**Cell Binding Properties of PfCCp Proteins**—In a concluding set of experiments, we wanted to investigate whether the adhesive properties of PfCCp proteins can be observed at the cellular level. Cell binding assays were performed on exflagellating *P. falciparum* cultures, using PfCCp-coated fluorescent latex beads. The recombinant proteins PfCCp1rp1-His6/SUMO, PfCCp2rp3-His6/SUMO, PfCCp3rp1-GST, and PfCCp3rp3-GST (for domain regions, see Fig. 3B) were bound to latex beads, followed by incubation with parformaldehyde-fixed activated or nonactivated gametocyte cultures (supplemental Fig. S4). The number of macrogametes, gametocytes, and erythrocytes that bound the PfCCp-coated latex beads were counted for 20 optical fields and compared with the total number of each of the investigated cell types. The cell binding assays revealed that the majority of latex beads adhered to macrogametes 15 min post-activation (supplemental Fig. S4). Approximately 25% of macrogametes bound to the beads (Fig. 4), with an average number of two beads/macrogamete. The binding of the beads to nonactivated gametocytes, macrogametes 6 or 24 h post-activation, and erythrocytes was significantly lower with only ~10% of cells having bound to latex beads. Similarly, there was no significant binding of the PfCCp-coated latex beads to macrogametes of PfCCp3-KO parasites (Fig. 4). These gene-disruptant parasites do not express any of the PfCCp proteins except for PfCCp4 (see above). Binding of latex beads to microgametes was never observed. As negative controls, the beads were covered with GST tag protein or the His6 tagged recombinant protein 5-Helix (21) and bound to ~10% of cells (Fig. 4). In summary, the recombinant PfCCp proteins bind to the surface of newly emerged PfCCp-expressing macrogametes, and these data provide the first evidence for the adhesive properties of macrogametes caused by PfCCp protein interactions.

**DISCUSSION**

During the last two decades a substantial number of proteins has been identified that are expressed in the sexual and mosquito stages of malaria parasites. The majority of these sexual stage proteins are initially expressed in the parasitophorous vacuole during gametocyte differentiation, and some are later exposed on the surface of emerged gametes and fertilized zygotes. Surface-associated proteins include Pf525 and Pf528, Pf548/45 and Pf547, Pf5230 and PfMR5, PfPeg3 and PfPeg4, and Pf14.744 and Pf14.748, as well as the six PfCCp proteins (2). Despite ongoing characterizations for most of the identified proteins, the functional basis for their expression within the parasitophorous vacuole of gametocytes, their exposure during emergence, and the role of the numerous adhesive motifs of these proteins remain unclear.

The PfCCp protein family represents prominent members of sexual stage proteins with their expression lasting for ~10 days. The proteins are expressed in early gametocytes during differentiation and are later present associated to the surface of macrogametes (11). For PfCCp1, PfCCp2, and PfCCp3, a partial release of protein during egress from the erythrocyte was reported, and the proteins later relocate surrounding exflagellation centers (7). The genes PfCCp2–PfCCp4 have been disrupted for functional characterization via single cross-over homologous recombination (7, 11). Surprisingly, gene-disruptant parasites revealed normal exflagellation and fertilization behavior, and oocysts developed in the midgut; however, in PfCCp2-KO and PfCCp3-KO lines, midgut sporozoite transition to the salivary glands was blocked (7). In contrast, PfCCp4-KO sporozoites were later present in the glands, leading to the assumption that PfCCp4 has a nonessential function for the malaria parasite (11). The striking difference in the predominant gametocyte-specific expression of the PfCCp proteins and the knock-out phenotype of some of the proteins during sporozoite formation hitherto is not known. We hypothesize that the molecular interactions of extracellularly exposed PfCCp proteins might mediate signaling events during fertilization that ultimately lead to sporozoite formation. The phenotypes for the newly generated PfCCp1-KO and PfFNPA-KO lines during mosquito transmission are currently under investigation.

The six proteins have also been studied in the rodent *P. berghei* system, where they are termed LAP proteins (9, 22). Transcript analysis revealed a predominant expression in *P. berghei* gametocytes for the six proteins, which was confirmed by transgenic parasites expressing green fluorescent protein driven by select *PbCCp/LAP* promoters (23). For functional characterization, loss of function mutants were generated for *PbCCp1/LAP2, PbCCp2/LAP4, PbCCp3/LAP1*, and *PbCCp4/LAP6* (22) as well as two double knock-outs, *PbCCp1/Ccp3* and *PbCCp1/Ccp4* (23). For all mutants, the formation of sporozoites in the midgut oocysts of parasite-infected mosquitoes was aborted (22, 23). Interestingly, sporulation of *PbCCp3/LAP1* (termed *PbSR* in this study) appears to be normal in *in vitro* assays, pointing to the involvement of mosquito factors in
the loss-of-function phenotype of this protein (24). Genetic cross studies with female-deficient or male-deficient parasites further indicated that the functional genes are inherited from female gametocytes (22). Green fluorescent protein fusions of PbCCp3/LAP1 (PbSR) showed protein expression in macrogametocytes and accumulation of these proteins in the ookinete crystalloid, suggesting a role in protein trafficking (24). However, no antibody-based protein expression analysis for the PbCCp/LAP proteins has so far been performed in P. berghei to support these results. In P. falciparum, PfCCp expression in the ookinete crystalloid was never observed (7, 11).

We recently reported that PfCCp1, PfCCp2, and PfCCp3 proteins are co-dependently expressed and that the abrogation of PfCCp3 in gene-disruptant gametocytes leads to the lack of PfCCp1 and PfCCp2 (10). At that time we hypothesized that the three proteins interact to form a protein complex. In the present study we address this hypothesis and described in detail the molecular interactions between the six members of the PfCCp protein family. First, we showed that all PfCCp proteins are co-dependently expressed and that the lack of one of the proteins leads to partial or complete loss of the other family members. We subsequently revealed that the six endogenous PfCCp proteins interact by forming protein complexes that were co-precipitated using a variety of PfCCp antibodies. Protein interactions were confirmed by direct protein binding of distinct adhesion domains using bacterially expressed recombinant proteins. This is the first time that such complexes involving interactions of multiple adhesive proteins are described for the sexual stages of malaria parasites.

The PfCCp proteins are comprised of multiple adhesive domains, whose remarkable architectures are conserved throughout the apicomplexan clade (8). The complex structure of these proteins is suggestive of roles in protein, lipid, and carbohydrate interactions. Affinity chromatography co-elution binding assays performed in this study indicate that particularly the two SR domains of PfCCp3 as well as the LCCL domains are involved in protein binding. The SR domain, present in a variety of metazoan organisms such as marine sponges and mammals (25), has been reported to be involved in protein-protein binding (26). In vertebrates, the SR domain is found in receptors on the surface of immune cells and in secreted proteins that are involved in host defense (25, 27, 28), regulation of the complement cascade (29), or binding and phagocytosis of invading pathogens (30). The LCCL domain, however, was so far only thought to be involved in binding of lipopolysaccharides (31,
membrane. However, surface-associated expression of the other PfCCp proteins is not dependent on PfCCp4, indicating that the PfCCp complex would be linked to the gametocyte by additional surface proteins.

In a concluding set of experiments we investigated whether the exposed PfCCp proteins are involved in cellular interactions. Using PfCCp-coated latex beads, we showed that PfCCp1, PfCCp2, and PfCCp3 specifically bind to newly formed macrogametes but not to macrogametes and zygotes 6 and 24 h post-activation. Furthermore, the binding of macrogametes is dependent on the presence of PfCCp proteins, as shown by adhesion studies on PfCCp3-KO parasites. In accord with these results, only the female macrogametes but not the male macrogametes or zygotes expose PfCCp proteins on their surface, and expression decreases within a few hours after emergence (11). We therefore conclude that the recombinant PfCCp proteins bind to endogenous PfCCp proteins that are exposed on the surface of the newly emerged macrogametes. Although not all macrogametes bind the PfCCp-coated latex beads, this might be due to the fact that the bacterially expressed recombinant proteins do not exhibit all adhesion domains of the respective full-length proteins and that they might also be subject to misfolding during expression, thus not exhibiting the full adhesive functions of the endogenous proteins.

In summary, the data presented here describe molecular interactions of sexual stage adhesion proteins. Our findings support the hypothesis that the six PfCCp proteins are components of a multi-protein complex, which is involved in cell adhesion events during gametogenesis and fertilization. We propose that the proteins are expressed in the parasitophorous vacuole of the differentiating gametocyte, where the complexes are initially assembled (Fig. 5). During the blood meal of the female mosquito, the gametocytes enter the mosquito midgut and become activated because of a shift in temperature and the presence of the mosquito-derived molecule xanthenic acid (2, 13, 14). After emergence of the activated gametocyte from the erythrocyte, the multi-protein complexes are exposed on the macrogamete surface, where they might be involved in adhesive interactions. It is tempting to speculate that the complexes mediate cell-cell contacts of macrogametes, e.g. binding of macrogametes to microgametes caused by interaction of select PfCCp proteins with Pf6230 or Pf648/45 on the microgamete surface.
mete surface. In this context, a recent study described the identification of the microgamete protein HAP2, which enables fertilization in *P. berghei* (40). Interestingly, HAP2 is essential for gamete fusion but not for the initial binding between the two mating partners, which appears to involve other adhesion proteins. Alternatively, the adhesive properties of the PfCCp multi-protein complexes might enable the microgamete to bind factors of the blood meal, e.g. factors of the human complement system, thus forming a protective shield that represents a barrier between the newly exposed gametes and the aggressive environment of the mosquito midgut. The possible involvement of other sexual stage adhesive proteins in the formation of the multi-protein complexes described here is currently being investigated in our laboratory.

Acknowledgments—We thank Dr. Catherine Lavazec for making the PfCCp1 disruption construct, Ludmilla Sologub for technical assistance, and Andres Abreu and Roland Frank for assisting with the construction of other sexual stage adhesive proteins in the formation of the PfCCp multi-protein complexes. During activation, the gametocyte egresses from the liver associated to the plasma membrane, where they assemble to multi-protein complexes, thus forming a protective shield that represents a barrier between the newly exposed gametes and the aggressive environment of the mosquito midgut.

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