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Short communication

Adhesion protein complexes of malaria gametocytes assemble following parasite transmission to the mosquito

Nina Simon [a,b,*], Andrea Kuehn [a,c], Kim C. Williamson [d], Gabriele Pradel [e]

[a] Research Center for Infectious Diseases, University of Würzburg, Josef-Schneider-Str. 2/D15, 97080 Würzburg, Germany
[b] Institute of Medical Biotechnology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Paul-Gordan-Str. 3, 91052 Erlangen, Germany
[c] ISGlobal, Barcelona Center for International Health Research (CRESIB), Hospital Clínic, Universitat de Barcelona, Barcelona, Spain
[d] Department of Biology, Loyola University Chicago, 6525 North Sheridan Road, Chicago, IL 60626, USA
[e] Division of Cellular and Applied Infection Biology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

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ABSTRACT

During differentiation in the human, the gametocytes of the malaria parasite *Plasmodium falciparum* display a remarkable number of adhesive proteins on their plasma membrane. These include the PfCCp protein family of six secreted proteins that assemble to multimeric protein complexes (MPCs) within the gametocyte parasitophorous vacuole. We now show that the PfCCp-based MPCs are linked to the gametocyte plasma membrane via interactions with Pfs230, a binding-partner of the GPI-anchored Pfs48/45. Upon onset of gametogenesis, which takes place after gametocyte uptake by blood-feeding mosquitoes, GPI-anchored Pfs25 joins the MPC, providing an additional link of its components to the plasma membrane. Gametogenesis also initiates cleavage of Pfs230 at its N-terminal site, resulting in its increased interaction with the MPC. Either lack of Pfs230 or impaired Pfs230 processing causes proteolysis of the PfCCp proteins and release from the MPC. Our data point to MPC assembly as a crucial step for sexual reproduction.

Multi-protein complexes (MPCs) are groups of two or more polypeptide chains linked by non-covalent protein–protein interactions. MPCs play important roles in regulating the activity and stability of polypeptides and are crucial for most cell biological processes. Moreover, protein complexes function in establishing cell–cell contacts and thus are of importance for interactions between pathogens and host cells. For intracellular pathogens such as malaria parasites, recognition, adhesion and invasion of host cells are essential steps during infection and are often mediated by protein–protein interactions. MPCs can also be found in the non-invasive, amotile gametocyte stages of *Plasmodium falciparum* (reviewed in [1]).

Gametocytes are dormant sexual precursor cells of the malaria pathogen, which differentiate within human red blood cells. Once taken up by a mosquito during the blood meal, the gametocytes become activated in the mosquito midgut by environmental stimuli. Within minutes upon activation, they exit the enveloping erythrocyte and transform into male and female gametes to initiate sexual reproduction and to continue the parasite's life-cycle in the insect vector (reviewed in [2]).

A remarkable feature of sexual reproduction of *P. falciparum* is the coordinated expression of numerous sexual stage proteins with adhesive properties. One subset of these proteins includes the cysteine motif-rich proteins Pfs230 and Pfs48/45 and the six PfCCp proteins, all of which are expressed during gametocyte maturation and which associate with the plasma membrane of the parasite within the parasitophorous vacuole. Some of these proteins are subsequently present on the gamete surface, but its expression usually ceases the latest during zygote formation.

The PfCCp proteins are a family of six secreted proteins with multiple adhesive modules, including a LCCL (*Limulus* clotting factor C; Cochlear protein 5b2; Lung gestation protein 1) adhesion domain shared by five of the six PfCCp proteins (thus also named LCCL-domain proteins). Orthologs of the PfCCp proteins were identified in other *Plasmodium* species as well as other Apicomplexan parasites, indicating an evolutionary conserved function across the apicomplexan clade (e.g. [3–6]). Previous studies demonstrated that the PfCCp proteins are expressed during gametocytogenesis and assemble to MPCs, which are localized in the lumen of the parasitophorous vacuole associated with the parasite plasma membrane (PPM) [7–9]. The MPCs remain associated with the surface of the developing macrogamete for a few hours following gametogenesis. The detailed mechanisms by which the secreted PfCCp proteins are linked to the PPM, however, remained unclear.

It was previously shown that Pfs230 is linked to the PPM of gametocytes and gametes via a molecular interaction with the GPI-anchored Pfs48/45 [10,11]. Further, we previously identified an interaction of PfCCp4 with Pfs230 [8]. Based on these data, we aimed to investigate the role of Pfs230 as a molecular coupler between the PfCCp-based

* Corresponding author at: Institute of Medical Biotechnology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Paul-Gordan-Str. 3, 91052 Erlangen, Germany.
E-mail address: nina.simon@fau.de (N. Simon).

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MPCs and the gametocyte PPM to determine the fate of the MPCs following gametogenesis.

We performed co-immunoprecipitation assays on lysates of mature non-activated gametocytes and of gametocytes at 30 min post-activation *in vitro* to detect potential MPC interaction partners by Western blotting (for domain structures of MPC proteins see Fig. S1). When we used antisera directed against any of the respective PfCCp proteins as bait, we were able to precipitate the other PfCCp proteins from gametocyte lysate (Figs. 1 and S2), confirming the previously reported molecular interactions between the PfCCp proteins [9]. For PfCCp1–PfCCp3, PfCCp5 and PfFNPA the full length proteins were immunoprecipitated, for PfCCp4 a previously described processed form running at ~75 kDa was pulled down. Also a previously described additional processed PfCCp2 protein of ~80 kDa was detectable [8,9]. While it was not possible to precipitate Pfs230 from lysate of non-activated gametocytes when using the anti-PfCCp1–PfCCp3 or anti-PfFNPA antisera, we detected Pfs230 via Western blotting when lysates of activated gametocytes were used in the assays (Figs. 1 and S2) (for experimental details, see the Materials and methods section of the Supplemental data). Noteworthily, Pfs230 is proteolytically processed upon gametocyte activation, resulting in a protein of ~300 kDa bound to the gamete surface [12]. The PfCCp-specific precipitates contained both the full-length and the processed forms of Pfs230 (Fig. 1). When antisera directed against PfCCp4 were used as bait, it was possible to precipitate full-length Pfs230 from the non-activated gametocyte lysate, while full-length and processed Pfs230 were present in the precipitates from lysates of activated gametocytes (Fig. 1). Vice versa, we detected the PfCCp proteins in lysates of activated gametocytes, when antisera against Pfs230 were used as bait in the assays. With the exception of PfCCp4, however, the PfCCp proteins were not detected in Pfs230-specific precipitates of lysates of non-activated gametocytes (Fig. 1). We further confirmed that Pfs230 binds to Pfs48/45. When antibodies against Pfs48/45 were used as bait in the assays, full-length Pfs230 was detected in the precipitate of lysate from non-activated gametocytes and both Pfs230 forms were detected in the precipitate from lysate of activated gametocytes (Fig. 1).

Female gametocytes are known to express the GPI-anchored protein Pfs25 in vesicular structures, from which the protein relocates to the PPM during gametogenesis [8,9]. After demonstrating that the PfCCp-based MPC components interact with Pfs230 upon gametocyte activation, we aimed to evaluate if these components might also interact with Pfs25. When antiserum against Pfs25 was applied in the immunoprecipitation assays on activated gametocyte lysates, it was possible to detect PfCCp1–PfCCp3 in subsequent Western blot analyses (Fig. 1). In all assays, antisera against the endoplasmic reticulum-associated protein Pf39 [13] were used for negative control and Pf39 was not detected in any of the precipitates (Fig. 1, S2). Similarly, when anti-Pf39 antisera were used as bait, neither the PfCCp proteins nor Pfs230 and Pfs25 were detected in the pull-downs (Fig. 1). For an additional negative control, we employed antisera against the highly abundant Pfs16 protein, which is located in the parasitophorous vacuole membrane of gametocytes [14], for immunoprecipitation. No prey proteins were detected in this control experiment, when antisera against PfCCp1–PfCCp3, PfFNPA, Pf39 and Pfs230 were used in Western blot analysis (Fig. S2).

To confirm the direct protein interactions between Pfs230 and selected PfCCps, we then performed co-elution binding assays. The recombinantly expressed region C of Pfs230 (see Fig. S1), fused with a maltose-binding protein (MBP) tag, was bound to a maltose column. When glutathione-S-transferase (GST)-tagged recombinant protein (rp) rp6 of PfCCp1 or rp3 of PfCCp3 was added to the columns, the proteins bound to the recombinant region C of Pfs230 and were subsequently co-eluted with the Pfs230 fragment, as detected by Western blotting using anti-GST antibodies (Fig. S3). As negative controls recombinantly expressed, GST-tagged rp1 of Pf39 or buffer alone was added to the columns and no co-eluted proteins were detected via Western blotting using anti-GST antibodies. Immunoblotting with anti-MBP antibodies confirmed that the recombinantly expressed region C of Pfs230 was eluted from the column (Fig. S3). In conclusion, the protein–protein interaction data indicated that the PfCCp-based MPC is linked to the gametocyte PPM via binding to Pfs230 and that the interaction intensifies upon gametocyte activation.

We then wished to know the fate of the PfCCp proteins in gametocytes, which lack the PPM-bound Pfs230 or which are chemically impaired in Pfs230 processing. For our studies, we used the Pfs230 gene-disruptant parasite line Pfs230-delta2 expressing an N-terminal 100 kDa fragment of the 360 kDa protein which is sequestered in a

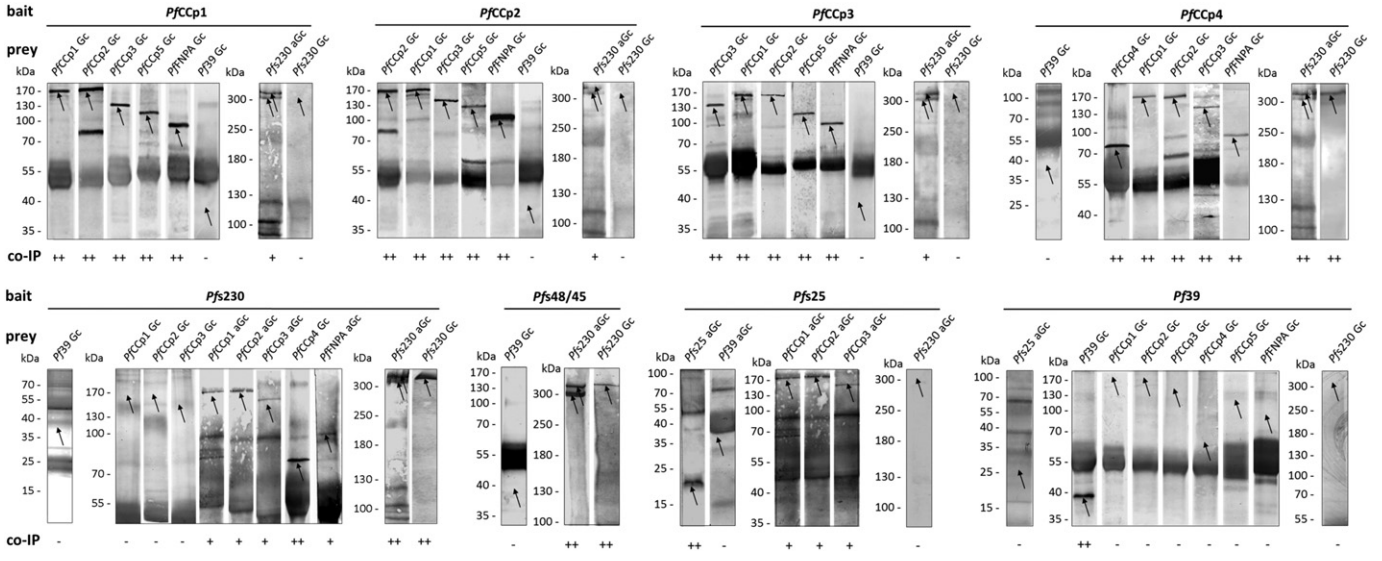

Fig. 1. Protein–protein interactions between MPC components. Lysates of WT strain NF54 mature non-activated gametocytes (Gc) and activated (aGc) gametocytes (30 min post-activation) were subjected to co-immunoprecipitation assay, using mouse or rabbit antisera against PfCCp1–PfCCp4, Pfs230, Pfs48/45 and Pfs25 (bait), followed by Western blot analysis using the same antisera as well as mouse antisera against PfCCp5 or PfFNPA to detect the precipitated proteins (prey) (for experimental details, see the Materials and methods section of the Supplemental data). Bands of immunoprecipitated proteins migrated at the expected molecular weights of 185 kDa (PfCCp1, PfCCp2), 143 kDa (PfCCp3), 180 kDa (PfCCp4), 119 kDa (PfCCp5), 100 kDa (PfFNPA), 360 and 300 kDa (Pfs230, unprocessed versus processed form). PfCCp4 migrated in a processed form of 75 kDa and an additional processed form of PfCCp2 migrated at 80 kDa. Mouse antisera directed against Pf39 (39 kDa) were used as a negative control for bait and prey in the assays. The intensities of the precipitated protein bands are indicated (++, strong; +, regular; −, negative). Smeared protein bands, migrating at ~55 and 20 kDa, resemble the heavy and light chains of the antibody used for precipitation.