Molecular & Biochemical Parasitology 201 (2015) 90–99

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Perforin-like protein PPLP4 is crucial for mosquito midgut infection by *Plasmodium falciparum*

Christine C. Wirth[a,1], Sandra Bennink[a,1], Matthias Scheuermayer[b], Rainer Fischer[c], Gabriele Pradel[a,c,*]

[a] *Cellular and Applied Infection Biology Section, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany*
[b] *Research Center for Infectious Diseases, University of Würzburg, Josef-Schneider-Str. 2/D15, 97080 Würzburg, Germany*
[c] *Fraunhofer Institute for Molecular Biology and Applied Ecology, Forckenbeckstr. 6, 52074 Aachen, Germany*

ARTICLE INFO

Article history:
Received 23 January 2015
Received in revised form 25 June 2015
Accepted 26 June 2015
Available online 9 July 2015

Keywords:
Plasmodium falciparum
Gametocyte
Ookinete
Perforin-like protein
Transmission
Mosquito
Malaria

ABSTRACT

The genomes of *Plasmodium* parasites encode for five perforin-like proteins, PPLP1-5, and four of them have previously been demonstrated to be involved in disruption of host cell barriers. We now show that the fifth perforin, PPLP4, is crucial for infection of the mosquito vector by *Plasmodium falciparum* parasites. PPLP4 is expressed in the blood and mosquito midgut stages in granular structures. In gametocytes, PPLP4 expression is specific to the female gender, while ookinetes show a PPLP4 localization at the apical pole. Gene disruption of *pplp4* results in no phenotypical change during blood stage replication, gametocyte development or gametogenesis, while mosquitoes fed with PPLP4-deficient gametocytes display a severe reduction in oocyst numbers, and an accumulation of ookinetes in the mosquito midguts was observed. In conclusion, we propose an essential role for PPLP4 in infection of the mosquito midgut, presumably by mediating ookinete traversal through the midgut epithelium.

1. Introduction

Perforins are secreted pore-forming proteins of eukaryotes, which possess a membrane attack complex/perforin (MACPF) domain, originally described in complement proteins of the terminal membrane attack complex and secreted granules of cytotoxic T lymphocytes and natural killer cells (reviewed in Ref. [1]). As a common feature, perforins are capable of switching between a soluble and a membrane-bound state and pore-formation is accomplished by assembly of the MACPF domains within target membranes.

Previously, a family of five perforin-like proteins, termed PPLP1-5, has been identified in malaria parasites via genome annotation [2]. For two of the proteins, PPLP1 and PPLP2, hemolytic activities during parasite egress from the host erythrocyte have been demonstrated. PPLP1 plays a role in merozoite release from the schizont during the erythrocytic replication cycle of *Plasmodium falciparum*, during which it is discharged from the merozoite micronemes in a calcium-dependent manner to mediate membrane rupture [3]. PPLP2 on the other hand supports the liberation of activated *Plasmodium berghei* or *P. falciparum* gametocytes from the enveloping erythrocyte at the onset of gametogenesis [4,5], which takes place in the mosquito midgut within a few minutes after the uptake of the gametocytes with the blood meal. Upon gametocyte activation, PPLP2 is calcium-dependently discharged from egress vesicles into the vacuolar space to perforate the erythrocyte membrane.

Three plasmodial perforins are further assigned to mediating the passage of the parasite infective stages through host cell epithelia. Besides its role in the erythrocytic replication cycle, PPLP1 (termed SPECT2 in this study) can be found in the micronemes of *P. berghei* sporozoites and is involved in breaching the liver sinusoidal cell layer prior to hepatocyte invasion [6]. Furthermore, PPLP3 (termed MAOP in this study) and PPLP5 were both shown to be essential for the traversal of the mosquito midgut epithelium by *P. berghei* ookinetes ([7,8]; reviewed in Ref. [9]).

To date, no functional analysis for the last member of the plasmodial perforin family, PPLP4, is available. Global proteomics analyses assigned the protein mostly to the ookinete stage of

Abbreviations: IFA, immunofluorescence assay; MACPF, membrane attack complex/perforin; MBP, maltose-binding protein; NMS, neutral mouse serum; p.a., post-activation; p.f., post-feeding; PPLP4(−), PPLP4-deficient; RT, room temperature; SEM, standard error of mean; SMFA, standard membrane feeding assay.

* Corresponding author at: Cellular and Applied Infection Biology Section, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany. Fax: +49 241 871062.
E-mail address: gabriele.pradel@molbiotech.rwth-aachen.de (G. Pradel).
[1] Authors contributed equally.

http://dx.doi.org/10.1016/j.molbiopara.2015.06.005

human as well as of rodent malaria parasites (www.plasmodb.org [10]). A previous attempt to generate PPLP4-deficient (PPLP4(−)) parasites in *P. berghei* resulted in the integration of the gene targeting construct into the genomic locus of a subset of parasites, indicating a non-essential role of PPLP4 in asexual blood stage growth; however, the generation of clonal lines failed [11]. We were now able to generate two clonal PPLP4(−) lines in the human malaria parasite *P. falciparum* and analyzed the role of PPLP4 for parasite transmission from the human to the mosquito.

2. Materials and methods

2.1. Gene IDs

The following PlasmoDB gene identification numbers (plasmodb.org; [10]) are assigned to the genes and proteins investigated in this study: Pfaldolase, PF3D7_1444800; PfAMA-1, PF3D7_1133400; PfCCp2, PF3D7_1455800; Pfs230, PF3D7_0209000; PfMSP1, PF3D7_0930300; Pfs25, PF3D7_1031000; Pfs28, PF3D7_1030900; PPLP4, PF3D7_0819400.

2.2. Parasite culture

P. falciparum asexual blood stage parasites and gametocytes of the wild-type (WT) strain NF54 (MR4 MRA-1000, BEI Resources Repository, NIAID, NIH) and the two PPLP4(−) lines PPLP4(−) 1C10 and PPLP4(−) 2D6 were cultivated *in vitro* in human blood group A+ erythrocytes as previously described [12,13]. All parasite stages were maintained in RPMI1640/Hepes medium (Gibco) supplemented with 10% heat inactivated human A+ serum, 50 μgml^{-1} hypoxanthine (Sigma–Aldrich) and 10 μgml^{-1} gentamicin (Gibco). For cultivation of PPLP4(−) parasites, the selection drug blasticidin (InvivoGen) was added in a final concentration of 5.4 μM. All cultures were strictly kept at 37 °C in an atmosphere of 5% O_2 and 5% CO_2 in N_2. Human erythrocyte concentrate and serum were purchased from the Department of Transfusion Medicine, University Hospital Aachen, Germany, or the Bavarian Red Cross in Würzburg, Germany. The donors remained anonymous and serum samples were pooled. To synchronize the asexual parasite blood stages, parasite cultures with 3–4% ring stages were centrifuged, the pellet was resuspended in five times pellet's volume of 5% sorbitol (AppliChem)/ddH_2O and incubated for 10 min at room temperature (RT) [14]. The cells were washed once with RPMI medium to remove the sorbitol, diluted to 5% vol. hematocrit with cell culture medium and further cultivated as described above. Gametocytes were enriched via Percoll gradient (GE Healthcare Life Sciences) as previously described [15]. Gametogenesis was induced by incubating mature gametocyte cultures in 100 μM xanthurenic acid dissolved in 1% vol. 0.5 M NH_4OH/ddH_2O for 15 min at RT [16,17].

2.3. Generation of PPLP4(−) parasite lines

PPLP4(−) parasites were generated via single cross-over homologous recombination using the pCAM-BSD vector [5,18]. To amplify a 531-bp gene fragment homologous to a region at the N-terminus of the MACPF domain of *pplp4* via PCR, PPLP4KO forward primer 5′-ATGGATCCCTAATCGTATGTTACTTGTTTATC-3′ and PPLP4KO reverse primer 5′-TAGCGGCCGCTTATTGGTACATAACTTCATTTGG-3′ were used. Ligation of the insert with the vector backbone was mediated by BamHI and NotI restriction sites (underlined). A NF54 WT culture with 4% ring stages was loaded with 60 μg of the disruption vector in transfection buffer via electroporation (parameters: 310 V 950 μF, 13 ms; Bio-Rad gene-pulser) as described [19]. Blasticidin (InvivoGen) was added to a final concentration of 5.4 μM, starting at 6 h post-transfection. A mock control was electroporated using transfection buffer without the disruption vector and cultured in regular medium. Blasticidin-resistant parasites appeared after 3–4 weeks. To check for successful plasmid integration into the *pplp4* gene locus, DNA of transfected parasites after 60–90 days of drug pressure was isolated using the NucleoSpin Blood Kit (Macherey-Nagel) according to the manufacturer's protocol and used as template in a diagnostic PCR. The following primers were used to confirm vector integration: PPLP4KO-5′-integration forward primer 5′-TTGCTTTAATAACTTTTGAACCATTT-3′ (1), PPLP4KO-3′-integration reverse primer 5′-ATACCACCAAAATGGGCAGA-3′ (2), pCAM-BSD forward primer 5′-TATTCCTAATCATGTAAATCTTAAA-3′ (3) and pCAM-BSD reverse primer 5′-CAATTAACCCTCACTAAAG-3′ (4) (for primer location, see Fig. S2A). After plasmid integration was confirmed, a mixed culture with >3% ring stages was diluted and transferred to a 96-well-plate. After three weeks of cultivation, clones were identified via Malstat assay [20] and subsequent diagnostic PCR was performed to confirm vector integration and the absence of the WT *pplp4* gene locus. Two clonal lines, PPLP4(−) 1C10 and PPLP4(−) 2D6, were isolated.

2.4. Recombinant protein expression

Two recombinant proteins of PPLP4 were bacterially expressed as fusion proteins with N-terminal maltose-binding protein (MBP)-tags. The respective gene sequences were amplified from *P. falciparum* genomic DNA using PPLP4rp1 forward primer 5′-ATGAATTCAAACCATTTTCAGCGTCTATGC-3′ and PPLP4rp1 reverse primer 5′-ATGAATTCTTACAGACCAGTGCTTTTTATATATCC-3′ resulting in a 903-bp gene fragment, as well as PPLP4rp2 forward primer 5′-GCGTAGGATCCAGCACTGGTCTGATTTTCGTT-3′ and PPLP4rp2 reverse primer 5′-TACGCCTGCAGTTAGGATTGGCTTTCACAAGGTTC-3′ resulting in a 634-bp gene fragment. Ligation of the PPLP4rp1 fragment with the pIH902-vector was mediated by EcoRI restriction sites (underlined). For ligation of PPLP4rp2 with the vector, BamHI and PstI restriction sites were used. Expression of recombinant proteins was done in *Escherichia coli* BL21 (DE3) RIL cells according to the manufacturer's protocol (Stratagene). The MBP-tagged fusion proteins were affinity-purified via amylose resin (New England Biolabs) as previously described [21] with following modifications of the procedure: pelleted bacteria were directly resuspended in lysis buffer containing protease inhibitor cocktail (complete® EDTA-free; Roche), incubated on ice for 20 min and homogenized by 4 min sonication (50 cycles/50% intensity). DNase treatment was not deployed. Amylose-bound fusion protein was eluted during batch purification according to the manufacturer's manual.

2.5. Diagnostic RT-PCR

Total RNA was isolated from synchronized trophozoite and schizont cultures of the NF54 WT strain, enriched immature (stage II–IV) or mature (stage V) gametocytes or gametocytes at 15 min p.a. using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. To remove genomic DNA contamination RNA preparations were treated with RNase-free DNase I (Qiagen), followed by phenol/chloroform extraction and ethanol precipitation. Photometric analyses of all RNA samples showed A260/A280 ratios higher than 2.1. Two micrograms of each total RNA sample were used for cDNA synthesis using the SuperScriptIII First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions. Transcript for *pplp4* (*pplp4.1*: 206 bp; *pplp4.2*: 201 bp) was amplified in 25 (gametocytes) versus 30 (trophozoites and schizonts) cycles using PPLP4RT1 forward primer 5′-GATCCAGGATTTAAAGAAGT-3′ and