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Transcript and protein expression analysis of proteases in the blood stages of *Plasmodium falciparum*

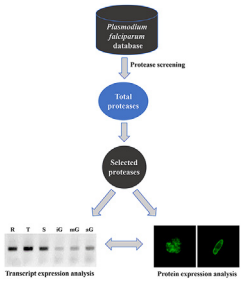
Tim Weißbach, Alexandra Golzmann, Sandra Bennink, Gabriele Pradel, Che Julius Ngwa*

Division of Cellular and Applied Infection Biology, Institute of Zoology, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany

HIGHLIGHTS

- *Plasmodium falciparum* database mining identifies about 148 putative proteases.
- Transcript expression profiling of selected proteases shows stage-specific expression of proteases.
- Protein expression analysis of selected proteases confirms their stage-specific expression.
- *P. falciparum* proteases represent ideal targets for stage-specific or multi-stage drugs.

GRAPHICAL ABSTRACT



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ABSTRACT

Proteases are crucial enzymes with varying roles in living organisms. In the malaria parasite *Plasmodium falciparum*, the role of proteases has been deciphered mainly in the asexual blood stages and shown to represent promising drug targets. However, little is known about their functions in the sexual blood stages, which are important for transmission of the disease from the human to the mosquito vector. Determination of their stage-specific expression during the malaria life-cycle is crucial for the effective design of multi-stage anti-malaria drugs aimed at eradicating the disease. In this study, we screened the *P. falciparum* genome database for putative proteases and determined the transcript and protein expression profiles of selected proteases in the plasmodial blood stages using semi-quantitative RT-PCR and indirect immunofluorescence assay. Database mining identified a total of 148 putative proteases, out of which 18 were demonstrated to be expressed in the blood stages on the transcript level; for 12 of these proteins synthesis was confirmed. While three of these proteases exhibit gametocyte-specific expression, two are restricted to the asexual blood stages and seven are found in both stages, making them interesting multi-stage drug targets.

1. Introduction

Malaria is the most deadly infectious tropical disease with an estimated 3.2 billion people at risk of being infected, causing 214 million new cases and approximately 438,000 deaths annually mainly in Africa (WHO, 2015). Malaria is caused by protozoan parasites of the genus *Plasmodium*; with the species *P. falciparum* being the most virulent agent responsible for malaria tropica

Abbreviations: EGF, epidermal growth factor-like domain; GPI, Glycosylphosphatidylinositol; TLCK, Tosyl-L-lysyl-chloromethane hydrochloride; TPCK, Tosyl phenylalanyl chloromethyl ketone.

* Corresponding author.
E-mail address: ngwa.che@bio2.rwth-aachen.de (C. Julius Ngwa).

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(reviewed in Greenwood et al., 2008).

Proteases represent a family of enzymes, classified into five major groups: cysteine, aspartic, serine, threonine and metallo proteases that possess different catalytic mechanisms with various active sites and divergent substrate specificities. In the malaria parasite *P. falciparum*, studies have demonstrated the role of proteases in key regulatory processes during the erythrocytic replication cycle including merozoite egress from schizonts, host cell invasion by merozoites, haemoglobin degradation and general catabolism (reviewed in Li et al., 2012).

The main task of proteases during the *P. falciparum* asexual replication cycle is the degradation of haemoglobin into single peptides. At first, the aspartate proteases plasmepsins 1, 2 and 4 as well as the histoaspartic protease, which become activated through the cysteine proteases falcipain 2, falcipain 2′ and falcipain 3 (*Pf*FP3) (Drew et al., 2008), degrade haemoglobin in globin polypeptides (Klemba et al., 2004). Metalloprotease falcilysin (*Pf*FLN) and dipeptidyl aminopeptidase 1 (*Pf*DPAP1) further degrade the remaining polypeptide into dipeptides (Klemba et al., 2004) which are finally cleaved by the M1-family alanyl aminopeptidase (*Pf*M1-AP) (Dalal and Klemba, 2007).

Three proteases were identified with particularly important roles in red blood cell (RBC) invasion and egress by merozoites. Subtilisin-like protease 1 (*Pf*SUB1) was the first identified serine protease in *P. falciparum* and is together with the serine-rich antigen 5 (*Pf*SERA5) and dipeptidyl aminopeptidase 3 (*Pf*DPAP3) crucial for RBC rupture during egress. Merozoites egress from the RBC in an explosive manner, which includes the sequential rupture of two membranes, the parasitophorous vacuole membrane (PVM) and the erythrocyte membrane (EM) (Chandramohanadas et al., 2011; Glushakova et al., 2010; Graewe et al., 2011). During merozoite egress the porous EM rapidly curls outwards, thereby efficiently dispersing the merozoites into the blood stream (Abkarian et al., 2011; Glushakova et al., 2005). Prior to merozoite egress from the RBC, plasmodial proteases are released from egress vesicles, the so-called exonemes, into the PV lumen. Among these is *Pf*SUB1, which is activated by *Pf*DPAP3. In the PV lumen *Pf*SUB1 proteolytically activates *Pf*SERA5, thereby releasing two peptides important for egress (Arastu-Kapur et al., 2008; Li et al., 2002; Okitsu et al., 2007; Yeoh et al., 2007; reviewed in Wirth and Pradel, 2012). Due to the crucial role of *Pf*SUB1 in the asexual blood stage replication cycle, further characterization studies were performed. The first crystallographic structure of *Pf*SUB1 was determined, which shows the calcium-dependence of *Pf*SUB1. It also revealed the presence of a solvent-exposed redox-sensitive disulphide bridge, unique among the subtilisin family, that likely acts as a regulator of protease activity in the malaria parasite (Withers-Martinez et al., 2014). Recent *in silico* studies have also revealed first inhibitory substances active against SUB1 in six different *Plasmodium* species (Brogi et al., 2016).

With the help of inhibitor studies, it has been demonstrated that protease inhibitors may represent key drugs for targeting the malaria parasite. Inhibitors of cysteine and serine proteases have consistently blocked EM rupture and merozoite invasion (Harris et al., 2005; Koussis et al., 2009). In addition, because the degradation of haemoglobin is vital for asexual blood stage parasite survival, the inhibition of this protease-mediated pathway blocks the release of haemoglobin and therefore kills the parasite (reviewed in Pérez et al., 2013).

Like for merozoite egress, proteases appear to play a major role in the egress of gametocytes from the host erythrocyte. It was shown that exflagellation of activated *P. berghei* and *P. falciparum* gametocytes can be blocked by the cysteine/serine protease inhibitors TLCK and TPCK (Rupp et al., 2008; Torres et al., 2005). Ultrastructural analyses on activated *P. falciparum* gametocytes subsequently demonstrated that TLCK and TPCK block the rupture of the EM. The cysteine protease inhibitor E64d, on the other hand, interferes with PVM rupture, suggesting that breakdown of the PVM and the EM are two independent events (Sologub et al., 2011; reviewed in Wirth and Pradel, 2012).

Despite the fact that proteases have been shown to be crucial for the asexual blood and the sexual stages, their expression in these stages has not been well characterized. Here, we provide a detailed combined transcript and protein expression profile of selected proteases in both the asexual and sexual blood stages of *P. falciparum*. Determination of the stage-specific expression of these proteases will open up more avenues for the effective design of stage-specific or multi-stage drugs against *P. falciparum.*

2. Materials and methods

2.1. Database mining

Proteases were initially identified using the *P. falciparum* peptidase database MEROPS (Rawlings et al., 2014) and sorted according to families and subfamilies. Subsequently, candidates were selected based on their dominant or exclusive expression in late stage gametocytes using RNA sequencing data taken from the database PlasmoDB (http://plasmodb.org/plasmo; Aurrecoechea et al., 2009). Additional proteases were chosen due to their roles in merozoite egress from the RBC. The database PlasmoDB was used for domain architecture determination of the selected proteases.

2.2. Parasite culture

Gametocyte non-producing F12 strain and gametocyte producing NF54 isolate of *P. falciparum* were cultivated in vitro in RPMI 1640 medium supplemented with 10% v/v heat-inactivated human serum as described (Ifediba and Vanderberg, 1981). Human erythrocyte concentrate and serum were purchased from the Department of Transfusion Medicine, University Hospital Aachen, Germany. Work with human blood was approved by the University Hospital Aachen Ethics commission, the donors remained anonymous and serum samples were pooled. Asexual blood stage cultures were repeatedly synchronized at 3–4% ring stages (Lambros and Vanderberg, 1979) and rings, trophozoites and schizonts were purified and stored in Trizol for RT-PCR. For enrichment of gametocytes, they were first treated with 50 mM N-acetyl glucosamine (GlcNac) for 5 days to kill the asexual blood stages, then maintained in normal culture medium without GlcNac until immature (stage II-IV) or mature stage V gametocytes were harvested and enriched by Percoll gradient purification (Kariuki et al., 1998). Gametocytes were activated by incubating mature gametocyte cultures in 100 μM xanthurenic acid dissolved in 1% v/v 0.5 M NH_4OH/ddH_2O for 20 min to 1 h at room temperature (RT). All cultures were maintained at 37 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2.

2.3. RNA isolation and RT-PCR

Total RNA was isolated from rings, trophozoites and schizonts (strain F12) and from enriched immature gametocytes (stage II-IV), mature gametocytes and gametocytes at 1 h post-activation (strain NF54), using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA preparations were treated with RNAse-free DNAse I (Qiagen) to remove gDNA contamination, followed by phenol/chloroform extraction and ethanol precipitation. Two μg of each total RNA sample were used for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions. Controls without reverse transcriptase were used to investigate potential gDNA