Molecular & Biochemical Parasitology 193 (2014) 101–109

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

The *Plasmodium* protein P113 supports efficient sporozoite to liver stage conversion *in vivo*

Vittoria Offeddu[a], Manuel Rauch[a], Olivier Silvie[b,c,d], Kai Matuschewski[a,e,*]

[a] *Max Planck Institute for Infection Biology, Parasitology Unit, 10117 Berlin, Germany*
[b] *Sorbonne Universités, UPMC Univ Paris 06, CR7, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), 75013 Paris, France*
[c] *INSERM U1135, CIMI-Paris, 75013 Paris, France*
[d] *CNRS ERL 8255, CIMI-Paris, 75013 Paris, France*
[e] *Institute of Biology, Humboldt University, 10117 Berlin, Germany*

ARTICLE INFO

Article history:
Received 20 August 2013
Received in revised form 1 March 2014
Accepted 3 March 2014
Available online 20 March 2014

Keywords:
Apicomplexa
Malaria
Plasmodium berghei
Experimental genetics
P113
Liver stage

ABSTRACT

Invasive stages of *Plasmodium* parasites possess distinct integral and peripheral membrane proteins that mediate host cell attachment and invasion. P113 is an abundant protein in detergent-resistant high molecular weight complexes in *Plasmodium* schizonts, but is unusual since expression extends to gametocytes and sporozoites. In this study, we tested whether *P113* performs important functions for parasite propagation in *Plasmodium berghei*. We show that pre-erythrocytic expression of *P113* displays key signatures of upregulated in infectious sporozoites (*UIS*) genes, including control by the liver stage master regulator SLARP. Targeted gene deletion resulted in viable blood stage parasites that displayed no signs of blood stage growth defects. *p113(−)* parasites propagated normally through the life cycle until mature sporozoites, but displayed defects during natural sporozoite transmission, leading to a delay to patency in infected animals. By comparative *in vitro* and *in vivo* analysis of pre-erythrocytic development and using a xeno-diagnostic test we show that ablation of *P113* results in lower sporozoite to liver stage conversion and, as a consequence, reduced merozoite output *in vivo*, without delaying liver stage development. We conclude that *p113* is dispensable for *Plasmodium* life cycle progression and plays auxiliary roles during pre-erythrocytic development.

1. Introduction

A hallmark of *Plasmodium*, the causative agent of malaria, is active and rapid target cell invasion by tailor-made extracellular stages of the otherwise obligate intracellular eukaryotic pathogen. Active entry is particularly important for invasion of erythrocytes, terminally differentiated cells no longer capable of receptor-mediated endocytosis. During blood and liver infection, *Plasmodium* parasites undergo a complex growth program, termed schizogony, and ultimately form merozoites, pear-shaped stages that specifically invade erythrocytes. While all extracellular parasites, *i.e.* merozoites, ookinetes, and sporozoites, are polarized and contain specialized apical organelles, they exhibit characteristic morphologies and express distinct surface proteins that bind to cognate receptors on target cells [1–3]. Gaining a better understanding of these parasite ligand and host cell receptor interactions remains a research priority and can inform vaccine discovery programs.

Since the identification of and successful immunization with the major merozoite surface protein MSP1 [4], numerous proteins on the merozoite surface and inside micronemes have been identified [3]. Micronemal proteins are only released upon environmental cues, such as host cell contact, and are considered important for parasite invasion. The first parasite–host interactions, including attachment and parasite positioning, are mediated by numerous integral merozoite surface proteins, which are typically membrane-anchored *via* a cleavable glycosylphosphatidyl inositol (GPI) moiety. Together with peripheral surface proteins they also largely determine the antigenic repertoire of *Plasmodium* merozoites and are targets of neutralizing antibodies [5].

In general, expression of merozoite antigens is under tight temporal expression control, in good agreement with their distinct roles in erythrocyte invasion [3,6]. A notable exception is the

Abbreviations: CSP, circumsporozoite protein; DRM, detergent-resistant membranes; GPI, glycosylphosphatidyl inositol; MSP1, merozoite surface protein 1; p113, protein of 113 kDa; UIS, upregulated in infectious sporozoites.

* Corresponding author at: Parasitology Unit, Max Planck Institute for Infection Biology, Charitéplatz 1, 10117 Berlin, Germany. Tel.: +49 30 2846 0535; fax: +49 30 2846 0225.
E-mail addresses: offeddu@mpiib-berlin.mpg.de (V. Offeddu), olivier.silvie@inserm.fr (O. Silvie), matuschewski@mpiib-berlin.mpg.de (K. Matuschewski).

http://dx.doi.org/10.1016/j.molbiopara.2014.03.002
0166-6851/

putative *Plasmodium falciparum* surface antigen P113 (PF3D7_1420700; gi: 124808655). P113 was isolated from synchronized blood stage parasites in raft-like membranes, so-called detergent-resistant membranes (DRM), which are highly enriched in GPI-anchored merozoite surface proteins [7]. Unlike other schizont DRM proteins, P113 was also detected in sporozoites [8] and early and mature gametocytes [9], indicative of ubiquitous expression. Subsequent biochemical work confirmed the presence of GPI anchors on many DRM proteins and permitted confident predictions of GPI modifications on several membrane proteins, including P113 [10]. Similar to MSP1, P113 is one of four abundant proteins in detergent-resistant high molecular weight complexes of *P. falciparum* schizonts, suggesting that it has critical roles in erythrocyte binding or invasion [11].

Experimental genetics in *P. falciparum* blood cultures indicated that most genes encoding GPI-anchored merozoite proteins, *e.g. MSP1* and *P92*, are refractory to targeted gene deletion [12–14]. Similarly, *P113* was reported as likely essential [3], but a systematic reverse genetics analysis has not been described yet. Accordingly, no potential function could be ascertained for P113 so far. Biochemical data indicate that P113 can bind to human spectrin and erythrocyte membrane protein 4.1 [15,16]. Both proteins are involved in the maintenance of the integrity of the red blood cell membrane and were shown to be required for normal *P. falciparum* growth in human erythrocytes [17]. These data, together with the recent suggestion that invasion of human red blood cells can be inhibited by high concentrations of anti-P113 peptide antisera [18], hint at potentially important roles of P113 in erythrocyte infection.

In this study, we attempted a systematic characterization of this protein by expression profiling and targeted gene deletion in the murine malaria model. Comparison of all *Plasmodium* orthologs with the founding member *P. falciparum* P113 shows considerable conservation with an overall amino acid sequence identity of ~34% (Fig. 1A and Suppl. Fig. 1). All orthologs are predicted to be GPI-anchored and to contain an amino-terminal signal peptide (Fig. 1A). We characterized *Plasmodium berghei* P113 (PBANKA_102250) employing a classical reverse genetics approach and show that absence of *P113* is compatible with normal parasite propagation in the intermediate and definitive hosts.

2. Materials and methods

2.1. Experimental animals

This study was carried out in strict accordance with the German 'Tierschutzgesetz in der Fassung vom 22. Juli 2009′ and the Directive 2010/63/EU of the European Parliament and Council 'On the protection of animals used for scientific purposes'. The protocol was approved by the ethics committee of the Berlin state authority ('Landesamt für Gesundheit und Soziales Berlin', permit number G0469/09). C57BL/6 mice were used for sporozoite infections. All other parasite infections were conducted in NMRI mice. Animals were from Charles River Laboratories.

2.2. Gene expression analysis by real-time quantitative RT-PCR

Total RNA was extracted from various life cycle stages of WT parasites, including synchronized blood stage schizonts, midgut and salivary gland sporozoites, and early and late liver stage parasites, using the RNeasy kit (Qiagen) as described [19]. RNA was reverse transcribed with the RETROScript kit (Ambion). Real-time PCR was performed on cDNAs using the StepOnePlus™ real-time PCR System and Power SYBR® Green PCR Master Mix (Applied Biosystems). Melting curve analysis confirmed the specificity of amplified products. *P113* expression levels were normalized to the constitutively expressed *GAPDH* gene. Each RT-PCR was performed in duplicate wells, and the whole series was repeated in an independent experiment with a new set of cDNAs. *P113* expression in *slarp(−)* sporozoites was performed in triplicates as above and normalized to GFP expression. Primers are listed in Table S1.

2.3. P113 gene targeting vectors and P. berghei transfection

A targeting construct for *P113* gene knockout was generated by inserting a 519 bp 5′UTR fragment and a 460 bp 3′UTR fragment amplified from *P. berghei* WT gDNA on either side of a *Tg*DHFR expression cassette in the standard transfection vector b3d [20]. After linearization of 5 μg DNA and transfection of synchronized *P. berghei* blood stage schizonts, recombinant parasites were selected *in vivo* by oral pyrimethamine. Successfully transfected parental lines were cloned *in vivo* by limiting dilution. Genotyping was done on gDNA by diagnostic PCR, RNA quantification, and Southern Blot. Amplification of *P113* transcripts from WT or *p113(−)* cDNA extracts was performed by RT-qPCR and *p113* expression was normalized to the constitutive *GAPDH*. Standard Southern Blot analysis was performed using the PCR DIG probe synthesis kit and the DIG luminescent detection kit (Roche). All primers are listed in Table S1.

2.4. Phenotyping and imaging of Plasmodium life cycle progression

The exponential phase of blood stage growth was analysed through intravenous injection of 10,000 infected erythrocytes into naïve recipient NMRI mice. Parasitemia was determined by microscopic quantification of daily Giemsa-stained blood films. *Anopheles stephensi* mosquitoes were raised under a 14 h light/10 h dark cycle at 28°C and 75% humidity. Blood-feeding and mosquito dissections were performed as described previously [21]. For every mosquito feeding experiment 10–15 female *Anopheles* mosquitoes were removed to enumerate the percentage of *P. berghei*-infected mosquitoes. Numbers of midgut- and salivary gland-associated sporozoites were determined at days 14 and 19 after feeding, respectively. For sporozoite gliding assay, sporozoites were liberated from infected salivary glands, added to 8-well slides (3000/well) and permitted to glide for 30 min. Immunofluorescence was done with monoclonal anti-*Pb*CSP antibody (3D11) and AlexaFluor488-conjugated goat anti-mouse IgG antibody. Hoechst (Invitrogen) was added to visualize nuclei. For the sporozoite cell traversal assay hepatoma cells (Huh7) were inoculated with 10,000 salivary gland sporozoites together with FITC-Dextran (0.5 mg/ml, Invitrogen). Dextran uptake during cell traversal was detected by FACS analysis. To quantify sporozoite attachment and cell invasion a two-colour invasion assay was performed as described [22]. Briefly, 10,000 sporozoites prepared in DMEM complete medium were added to cultured Huh7 cells for 90 min. Cells were fixed with 4% paraformaldehyde, followed by immunofluorescent assay using anti-*Pb*CSP antibody (3D11) and AlexaFluor546-conjugated goat anti-mouse IgG antibody to label extra-cellular sporozoites. Following cell permeabilization, intracellular parasites were detected with monoclonal anti-*Pb*CSP antibody (3D11) and AlexaFluor488-conjugated goat anti-mouse IgG antibody. Attached sporozoites were calculated from the total number of extra- and intracellular sporozoites. Liver stages were cultured *in vitro* and analyzed as described [19]. Briefly, hepatoma (Huh7) cells were incubated with 10,000 sporozoites and fixed at the indicated time points. To harvest liver stage merozoites, infected hepatoma cells were cultured for up to 72 h and merosomes collected from cell culture supernatants. Pre-patency and blood stage growth were assessed by daily microscopic examination of Giemsa-stained blood films. To measure parasite liver loads *in vivo*, 10,000 sporozoites were