Molecular & Biochemical Parasitology 166 (2009) 22–31

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Localisation and timing of expression of putative *Plasmodium berghei* rhoptry proteins in merozoites and sporozoites

Marta Tufet-Bayona[a], Chris J. Janse[b], Shahid M. Khan[b], Andrew P. Waters[c], Robert E. Sinden[a], Blandine Franke-Fayard[b,*]

[a] *The Malaria Centre, and the Division of Cell and Molecular Biology, Imperial College London, London SW7 2AZ, UK*
[b] *Leiden Malaria Research Group, Department of Parasitology, Centre for Infectious Diseases, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands*
[c] *Division of Infection and Immunity, Institute of Biomedical Life Sciences & Wellcome Centre for Molecular Parasitology, Glasgow Biomedical Research Centre, University of Glasgow, Scotland, UK*

ARTICLE INFO

Article history:
Received 14 November 2008
Received in revised form 22 January 2009
Accepted 17 February 2009
Available online 27 February 2009

Keywords:
Plasmodium berghei
Rhoptry
RON2
RAP2/3
PRP2
Merozoite
Sporozoite
Ookinete

ABSTRACT

Invasive forms of apicomplexan parasites contain secretory organelles (which may include micronemes, rhoptries or dense granules), the contents of which mediate invasion of host cells. Only few rhoptry proteins have been identified in *Plasmodium* and then only in merozoites and none in the sporozoite or ookinete. Epitope-tagged proteins (with either green fluorescent protein or C-MYC) were used to analyse the expression and cellular localisation of a known *Plasmodium* rhoptry protein (RAP2/3) and putative homologues of two *Toxoplasma* rhoptry proteins (rhoptry neck protein 2, RON2 and putative rhoptry protein 2, PRP2) at different stages across the life cycle. This analysis showed correct targeting to the merozoite rhoptries of GFP-tagged RAP2/3 and, for the first time, a distinct apical fluorescence pattern in sporozoites indicating a rhoptry location. In addition, tagged PBRON2 and PBPRP2 also show a merozoite rhoptry localisation similar to that of RAP2/3. RON2 is expressed in sporozoites and has the same timing of expression and location as RAP2/3. While PRP2 is also expressed in sporozoites, both its pattern of expression and location differ from RON2 and RAP2/3. None of the tagged proteins were detected in ookinetes, which is in agreement with the proposed lack of rhoptries in this third invasive form of *Plasmodium*. The analysis of tagged rhoptry proteins reveals new insights into the role of these proteins in host-cell invasion in different malarial 'zoites' and will facilitate more detailed studies into the role of rhoptries in establishing an infection of not only red blood cell but also the hepatocytes.

1. Introduction

Survival and successful transmission of the malaria parasite, *Plasmodium*, requires its invasive stages (merozoites, ookinetes and sporozoites) to recognize, bind, invade and develop within their respective host cells. These invasive stages are uniquely characterized by the presence of an apical complex that is common to all members of the phylum Apicomplexa. This complex contains specialized secretory organelles, which can include micronemes, rhoptries and dense granules; the contents of which are discharged sequentially upon invasion and are crucial for parasite invasion of the host cell and formation of a parasitophorous vacuole (further information can be found in the following review articles and references therein; [1–9]). Some surface proteins of these invasive stages as well as proteins released from the apical organelles during the process of invasion are exposed to the host's immune system, currently making them candidates for vaccine development. There is therefore considerable interest in the further identification and characterization of proteins from these organelles.

In the case of the malarial merozoite, invasion and apical protein secretion begins with the release of proteins from the microneme that are believed to be involved in the attachment of extracellular parasites to the host membrane, which is then followed by secretion of proteins from the rhoptries. Rhoptry secretion is initiated very rapidly after intimate contact of the parasite and the host cell, and is completed within a few minutes of invasion, implying that the contents of these organelles are vital in the establishment of the parasitophorus vacuole. In comparison to micronemal proteins, the role of rhoptry proteins is less well understood in apicomplexan parasites [5,10]. Approximately 20 proteins have been defined and examined in the rhoptries of *Plas-*

* Corresponding author. Tel.: +31 715265075; fax: +31 715266907.
E-mail address: bfranke@lumc.nl (B. Franke-Fayard).

0166-6851/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2009.02.009

modium merozoites [10–12]. Recently, a further 27 proteins in the rodent malaria parasite, *Plasmodium yoelii*, have been associated with merozoite rhoptries, based on a proteome analysis of merozoite rhoptry-enriched fractions [13,14], however, their functions remain unknown. Comparison of rhoptry proteins identified in *Toxoplasma gondii* with those in *Plasmodium* shows that many of these proteins are either *Toxoplasma* or *Plasmodium* specific, indicating they are highly adapted to the host cells that these parasites invade [6]. The *Plasmodium falciparum* rhoptry proteins include, among others, the high molecular-weight proteins that form a complex, RhopH1/Clag, Rhop2 and RhopH3 [15–18], and the low-molecular-weight proteins RAP1, RAP2 and RAP3 [19], these proteins have been implicated in invasion [20–22]. A proteomic analysis of purified rhoptries from *T. gondii* using mass-spectrometry resulted in the identification of 38 novel, putative rhoptry proteins [23], nine of which have homologues in *Plasmodium* and are unique to the Apicomplexa. Interestingly, four of the nine proteins conserved between *Plasmodium* and *Toxoplasma* were shown to be localized specifically to the duct-like neck portion of the *T. gondii* rhoptries, leading to their designation as rhoptry neck (RON) proteins. Upon invasion of the host cell, *T. gondii* RON2 is associated with both the micronemal apical-membrane antigen 1 (AMA1) and RON4, and they localize to the moving junction that is formed upon invasion [24]. The *Plasmodium* homologue of RON4 has recently also been shown to interact with AMA1 of *P. falciparum* [25]. In this study, to characterize the timing of expression and subcellular location of *Plasmodium* homologues of some *T. gondii* rhoptry proteins we have used molecular genetic approaches in the rodent malaria parasite *Plasmodium berghei* and have epitope-tagged 3 proteins. By tagging the known rhoptry protein RAP2/3 of *P. berghei* [19,26] we demonstrate the correct targeting of the protein to the rhoptries of merozoites. Using the same methodology, we have analysed the expression patterns and localisation of two putative rhoptry proteins, PbRON2 (the homologue of RON2 of *T. gondii*) and PRP2 (a conserved hypothetical protein, 'putative rhoptry protein 2'). We present evidence that both proteins are also targeted to the rhoptries of merozoites. In order to further examine the role of these proteins in invasion we examined the expression of these three tagged proteins through the complete life cycle of *Plasmodium*. Interestingly, all three proteins are also expressed in sporozoites and RAP2/3 and RON2 have a similar apical location, indicating a conserved and potentially associated role of RAP2/3 and RON2 in both merozoites and sporozoites invasion. We note with interest the absence of these proteins in the invasive ookinete, and discuss the implications of this finding on the biology of host-cell invasion and infection.

2. Materials and methods

2.1. *P. berghei* DNA sequences encoding (putative) rhoptry proteins

We analysed the *P. berghei* RAP2/3 rhoptry protein, which is encoded by a single copy gene located on chromosome 11 ([19]; incomplete *P. berghei* gene model PB301475.00.0; see Ref. [19] for the sequence of the complete gene). Additionally, we selected the orthologues of two *T. gondii* rhoptry proteins, 41.m01337 (TgTwinscan-2579) and 145.m00331 (TgTwinscan-0698; RON2) [23]. Using the DNA sequences of these *T. gondii* genes we searched the *P. berghei* gene sequence database (http://www.sanger.ac.uk) and were able by sequence homology to identify the homologous genes in *P. berghei*, i.e. PB000489.00.0 and PB000379.02.0, that we termed 'putative rhoptry protein 2' (PRP2) and RON2, respectively. Specifically, a BLASTN search with 41.m01337 identified the following genes from various *Plasmodium* species, *P. falciparum* (PF13_0116; score 189, e-value of $6e^{-46}$); *P. berghei* (PB000489.00.0; score 186, e-value of $4e^{-45}$) and *P. yoelii* (PY03873; score 185, e-value of $8e^{-45}$). The *prp2* gene is in the same syntenic context in all three *Plasmodium* species [27] being located on chromosome 13 in *P. falciparum* and chromosome 14 in both the rodent species. Further, the *prp2* gene shares 78.8% amino acid sequence identity with its *P. yoelii* orthologue and 58.1% with its *P. falciparum* orthologue. Similarly, BLASTN searches with *T. gondii ron2* gene (145.m00331) identified the following homologous genes in *Plasmodium*; *P. falciparum* (PF14_0495; score 308, e-value of $1e^{-86}$); *P. berghei* (PB000379.02.0; score 303, e-value of $2e^{-80}$) and *P. yoelii* (PY06813; score 247, e-value of $2e^{-63}$). The *Plasmodium ron2* genes were also in the same syntenic context in all three *Plasmodium* genomes, i.e. chromosome 14 in *P. falciparum* and on chromosome 13 in the rodent *Plasmodium* species. The *ron2* gene shares 77% amino acid sequence identity with its *P. yoelii* orthologue and 51% with its *P. falciparum* orthologue.

2.2. DNA constructs

Gene targeting DNA constructs were made to disrupt *rap2/3*, *prp2* and *ron2*. The sequences of these three genes (as well their corresponding up-, and down-stream sequences) were retrieved from the on-line *Plasmodium* genome database, www.plasmodb.org. Standard plasmid vectors (Supplemental Fig. 1) were designed for targeted gene disruption by double cross-over homologous recombination [28]. To replace the protein coding sequences of the target genes with the selection cassette containing the pyrimethamine-resistant *dhfr/ts* of *T. gondii*, we cloned the 5′ and 3′ flanking regions of the genes of interest up- and down-stream of the selection cassette of pL0001 or pL0037. To obtain pL0037, first the *Asp*718 site of pL0035 was destroyed by Klenow filling and then the selection cassette was cloned as a *Hind*III/*Eco*RV fragment into pL0001; plasmids pL0001 and pL0035 are available from MR4; www.mr4.org/). Briefly stated: for one candidate gene, to generate a *RON2* disruption vector, a 747-bp upstream region and a 628-bp downstream region were amplified from *P. berghei* genomic DNA using primer-pairs L3027/3028 and L3149/3150, respectively (see Supplemental Table 1 for the sequence of the primers). The PCR products were digested with *Asp*718 and *Hind*III, or *Eco*RI and *Xba*I, respectively, and ligated into plasmid pL0001 yielding the targeting plasmid pL1277. See Supplemental Fig. 1 and Supplemental Table 1 for further details of targeting plasmids for *ron2* (pL1277), *RAP2/3* (pL1427) and *PRP2* (pL1276) and the sequence of the primers. The targeting vectors for the disruption of *ron2* and *prp2* were linearized using *Asp*718 and *Xba*I and *rap2/3* with *Asp*718 and *Eco*RI. Transfection experiments were performed at least two times, as previously described [28].

Epitope-tagging constructs were made to tag RAP2/3, PRP2 and RON2. See SOM Table 1 for the sequence of the primers used to generate these constructs. To generate construct RAP2/3-GFP, the complete rap2/3 coding sequence (accession number: XM_669159.1) was amplified from *P. berghei* genomic DNA by PCR with 5′ primer L1734 (containing *BamH*I restriction sites) and 3′ primer L1735 (replacing the stop codon with an *Nco*I site). The *rap2/3* amplicon was ligated into *BamH*I–*Nco*I digested plasmid pL0025, generating the pAMA1-RAP2/3-GFP plasmid. Then the *BamH*I sites of pL0007, flanking the *hdhfr* gene were destroyed by cloning the *hdhfr* gene as *bgl*II PCR product using primers 886 and 887 to obtain pL0007-BglII. Subsequently, the *tgdhfr/ts* selectable cassette of pL0001 was exchanged with the *hdhfr* selectable cassette of pL0007-BglII by restriction with *Mun*I/*Sap*I to create pL0001-hdhfr. Finally, the *rap2/3-egfp-3′utr-pbdhfr* fragment (containing *rap2/3* in-frame with *gfp* and followed by the 3′UTR of *pbdhfr*) was