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Plasmodium RON12 localizes to the rhoptry body in sporozoites

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ABSTRACT

Invasion of host cells by apicomplexan parasites is mediated by proteins released from microneme, rhoptry, and dense granule secretory organelles located at the apical end of parasite invasive forms. Microneme secreted proteins establish interactions with host cell receptors and induce exocytosis of the rhoptry organelle. Rhoptry proteins are involved in target cell invasion as well as the formation of the parasitophorous vacuole in which parasites reside during development within the host cell. In *Plasmodium* merozoites, the rhoptry neck protein (RON) complex consists of RON2, RON4, and RON5, and interacts with apical membrane antigen 1 (AMA1) as a critical structure of the invasion moving junction. PfRON12 is known to localize to the rhoptry neck of merozoites, but its function remains obscure. The roles of RON proteins are largely unknown in sporozoites, the second invasive form of *Plasmodium* which possesses a conserved apical end secretory structure. Here, we confirm that RON12 is expressed in the rhoptry neck of merozoites in rodent malaria parasites, whereas in contrast we show that RON12 is localized to the rhoptry body in sporozoites. Phenotypic analysis of *Plasmodium berghei ron12*-disrupted mutants revealed that RON12 is dispensable for sporogony, invasion of mosquito salivary glands and mouse hepatocytes, and development in hepatocytes.

1. Introduction

Apicomplexan parasites, including *Plasmodium* the causative agent of malaria, are characterized by conservation of three apical secretory organelles - namely, micronemes, rhoptries, and dense granules - that are required for the infection of new host cells. In *Plasmodium*, micronemes exist in all invasive parasite forms, merozoites, ookinetes, and sporozoites, but rhoptries are found only in merozoites and sporozoites which invade host cells with the concomitant formation of a parasitophorous vacuole (PV) [1]. The rhoptries are the most prominent of the secretory organelles in *Plasmodium*. Rhoptries in merozoites are composed of two distinct regions, an apical duct known as the rhoptry neck and a larger bulb-like rhoptry body; with each compartment containing a distinct protein constituent. Invasive forms of apicomplexan parasites sequentially secrete proteins from the apical organelles in a highly regulated manner during invasion of host cells (reviewed in [2]). For example, the rhoptry neck proteins (RONs) RON2, RON4, and RON5 are secreted from the apical end during invasion of *Plasmodium* merozoites and *Toxoplasma* tachyzoites, and transferred as a complex to the host plasma membrane at the tight junction formed between parasites and their host cells [3,4]. It has been demonstrated that the RON complex interacts with another secretory protein, apical membrane protein 1 (AMA1) on the parasite membrane, which is crucial for parasite invasion of target cells [5–11]. Subsequently, rhoptry body proteins are secreted after tight junction formation or completion of invasion, which are then transferred to the parasitophorous vacuole membrane (PVM) [2]. In *Plasmodium falciparum,* more than 30 proteins are classified as rhoptry proteins, of which rhoptry neck proteins are highly conserved among apicomplexan parasites as described above [11–13]. In contrast, rhoptry body proteins are more diverse across genera; for instance, many protein kinases are demonstrated to be transferred to the PVM after *Toxoplasma* tachyzoites invasion and involved in PVM maintenance, while no kinases localized to the rhoptry body in *Plasmodium* have been reported [2,14,15].

Sporozoites are another invasive form in the *Plasmodium* lifecycle, which first invade mosquito salivary glands, and then infect hepatocytes in

Abbreviations: RON, rhoptry neck protein; PV, parasitophorous vacuole; AMA1, apical membrane antigen 1; DHFR, dihydrofolate reductase; IFA, indirect immunofluorescence assay; IEM, immunoelectron microscopy

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mammalian hosts with the formation of a PVM. Sporozoites also possess apical complex rhoptries. The hypothesis that merozoites and sporozoites partly share mechanisms for invasion of target cells is supported by findings that RON2, RON4, and RON5 are also expressed in sporozoites; and, moreover, that RON4 is secreted prior to invasion of hepatocytes [16–19]. It is possible that PVM formation in hepatocytes might be mediated by sporozoite-specific mechanisms, in addition to mechanisms conserved with blood stage merozoites. This is suggested by the observation that the liver-stage specific exported proteins UIS3 and UIS4 ("upregulated in infective sporozoites" 3 and 4) are transferred to the PVM and have crucial roles [20,21]. Until recently the roles of rhoptry proteins in sporozoites were largely unknown, as most rhoptry proteins are essential for parasite proliferation during the intra-erythrocytic stage and therefore it is not possible to generate gene-modified parasites disrupted in rhoptry protein production. RON4 was solely demonstrated as having a crucial role for sporozoite infection of hepatocytes using a conditional gene silencing system in *P. berghei* [17]. Here, we focused on RON12, reported as a *Plasmodium* specific protein, despite its localization to the rhoptry neck in merozoites [22] and the typical broader conservation of rhoptry neck proteins in apicomplexans such as *Toxoplasma*. Among the *Plasmodium* genus, however, the amino acid sequences of RON12 orthologues are highly conserved. RON12 has been demonstrated as non-essential for the survival of blood-stage parasites under experimental conditions [22]. In this study, we demonstrate that RON12 is also expressed in sporozoites and that it is localized to the rhoptry body, rather than to the rhoptry neck as described for its localization pattern in merozoites. Targeted gene disruption revealed that RON12 is dispensable for sporogony, sporozoite invasion of mosquito salivary glands and mammalian hepatocytes, and also for liver stage parasite maturation inside hepatocytes.

2. Materials and methods

2.1. Parasites and mice

ICR female mice, 6–8 weeks old (CREA Japan, Tokyo, Japan) at the time of primary infection, were kept in a room with a temperature of 24 °C under a 12 h light/12 h dark cycle. Cryopreserved *P. yoelii* 17XNL and *P. berghei* ANKA infected erythrocytes were intraperitoneally injected into female ICR mice, pretreated 3 days prior with 200 µl of 6 mg/ml phenylhydrazine (Wako Pure Chemical, Osaka, Japan) in 1 × PBS. Infected blood (around 5% parasitemia) was collected by cardiac puncture at day 4 after infection. Schizont-rich fractions were collected by NycoPrep (Progen Biotechnik, Heidelberg, Germany). For mosquito feeding experiments, infected ICR mice were fed to *Anopheles stephensi* (SDA500 strain) mosquitoes and fully engorged mosquitoes were selected and kept at 20 or 24 °C until dissection. At day 10–14 post-feeding, the number of oocysts was examined to determine the prevalence. Sporozoites were collected from midguts or salivary glands by dissection at the indicated day post blood meal and the sporozoite number was counted. To determine sporozoite infectivity to the mouse liver, 30,000 sporozoites collected from salivary glands of infected mosquitoes at day 21–24 post-feeding were injected intravenously into ICR female mice and the parasitemias were monitored every 12 h from day 2 to day 8 post-injection. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Ehime University and the experiments were conducted according to the Ethical Guidelines for Animal Experiments of Ehime University.

2.2. Targeted gene disruption of pbron12

Pbron12-disrupted parasites (ΔPbRON12) were generated by double crossover homologous recombination using the gene disruption vector pL0006 available from BEI Resources. To replace the endogenous *pbron12* (PBANKA_0501400) genomic locus with a pyrimethamine-resistant selectable marker containing the human dihydrofolate reductase gene (*dhfr*), two homologous recombination cassettes (*pbron12*–5: −970 to +53 bp and *pbron12*–3: +466 to +1257) were inserted into pL0006 at both sides of the drug-resistant cassette. The DNA fragments of *pbron12*–5 (1023 bp) and *pbron12*–3 (792 bp) were amplified from PbANKA genomic DNA (gDNA) using PbRON12-KO5-F-*Hin*dIII (5′-CCCAAGCTTGTTGTTTGGATAATTGAGTTGCGT-3′) and PbRON12-KO5-R-*Bgl*II (5′-GAAGATCTACAACCAATATGCATACCAAAACC-3′), PbRON12-KO3-F-*Kpn*I (5′-GGGGTACCGAAAATGGAGAAATATCTGAATCC-3′) and PbRON12-KO3-R-*Nhe*I (5′-CTAGCTAGCGACAACGTATATCTATACATA TGA-3′), respectively. The fragments were inserted into *Hin*dIII and *Bgl*II sites or KpnI and NheI sites, respectively. The plasmid was digested with HindIII and NheI before transfection. Enriched schizonts of PbANKA were transfected with 10 µg of digested plasmid by electroporation using Nucleofector (Lonza Japan Ltd. Tokyo, Japan) with a human T cell solution and the U-33 program and then parasites were selected with pyrimethamine [23]. The integration of the target DNA fragment was determined by PCR, using PbRON12 KO check-F (F1: 5′-CAGGGTTAACATTTTGCTGTGGTT-3′) and PbRON12 KO check -R (R2: 5′-TTGAGGGGTGAGCATTTAAAGCAC-3′). Wild type parasites were detected by PCR using PbRON12 KO check-F and PbRON12 KO check WT-R (R1: 5'-CATGCTCCTTAACAGTATATTCAAC-3′). ΔPbRON12 parasites were cloned by limiting dilution.

2.3. Recombinant protein expression and antisera production

A DNA fragment encoding full-length of PyRON12 (PY00202) except an N-terminal signal peptide (amino acid positions (aa) 26–255) was amplified from PyWT blood stage cDNA by PCR, using primer pairs PyRON12 F-*Xho*I (5′- CTCGAGAGAATGCATAAGCCAGTTGAGTA TAC-3′) and PyRON12 R-*Bam*HI (5- GGATCCTTATTCGGTCAAATCTG ACACATTTTC-3′). The amplified PyRON12 DNA fragment was inserted between the XhoI and BamHI sites of plasmid pEU-E01-HisGST(TEV)-N2 (CellFree Sciences, Matsuyama, Japan). The PyRON12 recombinant protein tagged by His/GST at the N-terminus was expressed using a wheat germ cell-free system (CellFree Sciences) [24,25]. Expressed recombinant PyRON12 was captured by a glutathione-Sepharose 4B column (GE Healthcare, Piscataway, NJ, USA) and eluted with elution buffer (40 mM reduced glutathione, 50 mM Tris-HCl, 300 mM NaCl, 200 mM imidazole, 2% glycerol, pH 8.0).

To generate antisera against PyRON12 (full-length with His/GST-tag), a Japanese white rabbit was immunized subcutaneously with 250 µg of purified recombinant protein with Freund's complete adjuvant, followed by two immunizations using 250 µg of purified recombinant protein with Freund's incomplete adjuvant. All immunizations were done at 3-week intervals and antisera were collected 14 days after the last immunization (Kitayama labes Co. Ltd. Ina, Japan). To purify specific anti-PyRON12 antibodies, 1 ml HiTrap NHS-activated HP column (GE Healthcare) was coupled with recombinant His/GST tagged PyRON12 as described by the manufacturer. After passing through rabbit antisera, the column was washed with 20 mM phosphate buffer, pH 7.0 before elution. The bound PyRON12 specific antibodies were eluted with 0.1 M glycine–HCl, pH 2.7, and immediately neutralized with 1 M Tris, pH 9.0.

2.4. Western blotting analysis

Parasite lysates from enriched schizonts or sporozoites collected from midguts of infected mosquitoes were mixed with SDS-PAGE loading buffer, and then boiled at 97 °C for 5 min, followed by separation by electrophoresis on a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan) under a non-reducing condition. Proteins were transferred to 0.2 µm polyvinylidene fluoride membranes (ATTO). Membranes were incubated with Blocking One (Nacalai tesque, Kyoto, Japan) followed by immunostaining with affinity purified anti-PyRON12 primary antibodies (1 µg/ml). Pre-immune rabbit serum (1: 1000 dilution) was used as a negative control. PbHSP70 was detected using rabbit anti-PbHSP70 antiserum (1:10000 or 100,000 dilution) as a loading control. The membranes were then probed with HRP-