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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-

conjugated goat anti-rabbit IgG antibodies (GE Healthcare) and visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) on a LAS 4000 luminescent image analyzer (GE Healthcare). The relative molecular masses of the proteins were estimated with reference to Precision Plus Protein Standards (Bio-Rad, Hercules, CA, USA).

2.5. Indirect immunofluorescence assay (IFA) and immunoelectron microscopy (IEM)

Infected blood smears or sporozoites were fixed on glass slides with ice-cold acetone for 5 min and blocked with PBS containing 5% nonfat milk at 37 °C for 1 h. They were then incubated with affinity purified anti-PyRON12 antibodies at 1 μg/ml at 37 °C for 1 h, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen) as secondary antibodies (1:500 dilution) at 37 °C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/ml) mixed with a secondary antibody solution. Slides were mounted in ProLong Gold Antifade reagent (Invitrogen) and observed using a fluorescence microscope (Axio observer z1, Carl Zeiss, Oberkochen, Germany).

For immunoelectron microscopy, schizont-enriched infected erythrocytes or salivary glands from infected mosquitoes were fixed for 30 min on ice in a mixture of 1% paraformaldehyde–0.2% glutaraldehyde in 1× HEPES buffer (pH 7.05). Fixed specimens were dehydrated and embedded in LR-White resin (Polysciences Inc., Warrington, PA). Ultrathin sections on a grid were blocked in PBS containing 5% non-fat milk and 0.01% Tween 20 (PBS-MT), then incubated at 4 °C overnight with purified anti-PyRON12 antibodies (1:1000 dilution) in PBS-MT. After washing with PBS containing 0.4% BlockAce (DS-Pharma Co, Japan) and 0.01% Tween 20 (PBS-BT), the grids were incubated at 37 °C for 1 h with goat anti-rabbit IgG conjugated to 15 nm gold particles (BBI International, Minneapolis, MN) diluted 1:20 in PBS-MT, and rinsed with PBS-BT followed by water as described [26]. Ultrathin sections were stained with uranyl acetate and lead citrate. Samples were examined with a transmission electron microscope (JEM-1230, JEOL, Japan).

2.6. IFA of parasite infected hepatocytes

HepG2 cells, a human hepatoma cell line obtained from RIKEN Cell Bank, were seeded in an 8-well chamber slide (Thermo Fisher Scientific, San Jose, CA) and cultured for 2 days in RPMI1640 media containing 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Wako Pure Chemical) at 37 °C in the presence of 5% CO_2. Approximately 10 thousand *P. berghei* sporozoites collected from salivary glands were inoculated to HepG2 cells and incubated until cells were fixed with 10% formalin at indicated time points. Cells were treated with methanol for 10 min at room temperature and blocked with 5% skim milk-PBS for 30 min at 37 °C. They were then incubated over-night with affinity purified anti-PyRON12 antibodies (1 μg/ml) and monoclonal antibodies against Pb circumsporozoite protein (PbCSP, 1:5000, MRA-100; BEI Resources) at 4 °C, followed by incubation for 30 min with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen) as secondary antibodies (1:500 dilution) at 37 °C. Nuclei staining and observation were performed as described above.

3. Results and discussion

3.1. RON12 is expressed in P. yoelii and P. berghei merozoites and localizes to the rhoptry neck

To characterize the expression and localization of PyRON12, we raised rabbit antibodies against nearly full-length recombinant PyRON12 protein (amino acids 26–255; lacking the N-terminal signal peptide) expressed by the wheat germ cell-free protein synthesis system (Fig. S1). To evaluate the reactivity of the antibodies against parasite-derived PyRON12, Western blot analysis was performed using schizont-enriched *P. yoelii* parasites. PyRON12 was detected as a major band of approximately 28 kDa under a non-reducing condition as indicated by an arrow (Fig. 1A, left). This size approximately corresponds to its calculated molecular weight and is smaller than that of *P. falciparum* RON12 (PfRON12, 37 kDa) [22]. Anti-PyRON12 antibodies were used to investigate the subcellular localization of RON12 by IFA (Fig. 1A, right). A punctate staining pattern was observed in schizonts of *P. yoelii*, consistent with an apical localization of RON12. These findings indicate that anti-PyRON12 antibodies specifically recognize parasite PyRON12. Since the amino acid sequence identity between PyRON12 and PbRON12 is 92%, we then investigated whether anti-PyRON12 antibodies also react to *P. berghei* RON12 (PbRON12). In Western blot analysis using schizont-enriched *P. berghei* parasites, the anti-PyRON12 antibodies mainly recognized a protein of approximately 28 kDa, which correlates with the calculated molecular weight of PbRON12 (Fig. 1B, left). In IFA using *P. berghei* schizonts as an antigen, anti-PyRON12 antibodies showed punctate reactivity to schizonts (Fig. 1B, right). These results indicate that anti-PyRON12 antibodies also recognize PbRON12. Since PfRON12 has been reported to localize to the rhoptry neck of merozoites [22], detailed subcellular localization of RON12 in *P. yoelii* and *P. berghei* was investigated by IEM using schizont-enriched specimens. Gold particles indicating the presence of PyRON12 were observed in the rhoptry neck region and not in the rhoptry body in *P. yoelii* merozoites (Fig. 2A, B). IEM using *P. berghei* schizont-enriched samples also showed that the anti-PyRON12 antibodies reacted with the antigen located in the neck portion of rhoptries in merozoites (Fig. 2C, D). These observations confirmed that RON12 is localized to the neck of rhoptries in merozoites.

3.2. RON12 is localized to the rhoptry body in sporozoites

A limited number of rhoptry proteins such as RON2, RON4, RON5, and RAP2/3 have been described in both merozoites and sporozoites [16–18,27,28]. Although RON12 was reported to localize to the rhoptry neck of merozoites [22], the expression and subcellular localization of RON12 in sporozoites remains unknown. Therefore, we examined the expression of RON12 in *P. yoelii* and *P. berghei* sporozoites using oocyst-derived sporozoites. As shown in Fig. 3A, anti-PyRON12 antibodies detected PyRON12 and PbRON12 in sporozoites as the major band with the same molecular weight of 28 kDa as that of merozoites. To investigate the localization of RON12 in sporozoites, IFA was performed using anti-PyRON12 antibodies. Fluorescent signal was observed in the apical portion of sporozoites in both *P. yoelii* and *P. berghei* (Fig. 3B). Subcellular localization of RON12 in sporozoites collected from salivary glands was further analyzed using IEM. RON12 was observed exclusively in the rhoptry body and no reaction was detected in the narrow neck portion of rhoptries in both *P. yoelii* (Fig. 4A, B) and *P. berghei* (Fig. 4C, D). No reactivity was detected in micronemes. These data clearly demonstrate that RON12 is expressed in rhoptries of sporozoites; however, unlike the expression pattern observed in merozoites, the fine localization is within the body rather than the neck of rhoptries.

Some rhoptry body proteins in merozoites, such as RON3, RAMA, and RAP1 have been shown to be transferred to the PV space after parasite invasion of erythrocytes [29–32]. Despite its localization to the rhoptry neck, it was reported that RON12 is also transferred to the PV space after merozoite invasion. Therefore, RON12 localization in infected hepatocytes was further analyzed by IFA. RON12 remains at the apical end in sporozoites shortly after invasion of HepG2 cells (human hematoma cells; Figs. 5, 2h), suggesting that RON12 is not secreted prior to invasion, which is like PfRON12 in merozoites. Within sporozoites transforming to the early liver stage, RON12 is localized in the cytoplasm as spots (Figs. 5, 6h and 24 h). At the late liver stage,

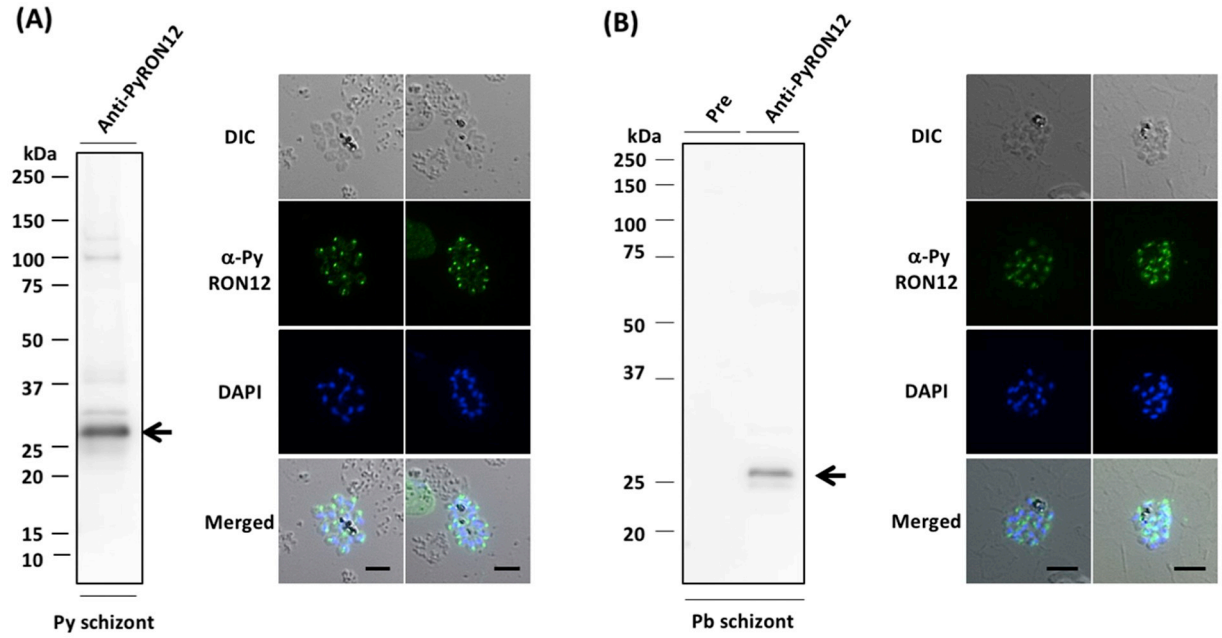

Fig. 1. Reactivity of anti-PyRON12 antibodies against *P. yoelii* and *P. berghei* schizont samples.
Reactivity of anti-PyRON12 antibodies against *P. yoelii* schizonts (A) and *P. berghei* schizonts (B). Schizont-enriched parasite extracts of *P. yoelii* or *P. berghei* were separated by SDS-PAGE under non-reducing conditions and subjected to immunoblotting with anti-PyRON12 antibodies at 1 μg/ml. Specific bands corresponding to PyRON12 and PbRON12 are indicated by arrows. Pre-immune rabbit serum (Pre) was used as a negative control. Localization of PyRON12 and PbRON12 in schizonts was demonstrated by IFA. RON12 was specifically detected as a punctate pattern in mature schizonts using anti-PyRON12 antibodies at 2 μg/ml in both *P. yoelii* and *P. berghei*. Nuclei were stained with DAPI (blue). Scale bars, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

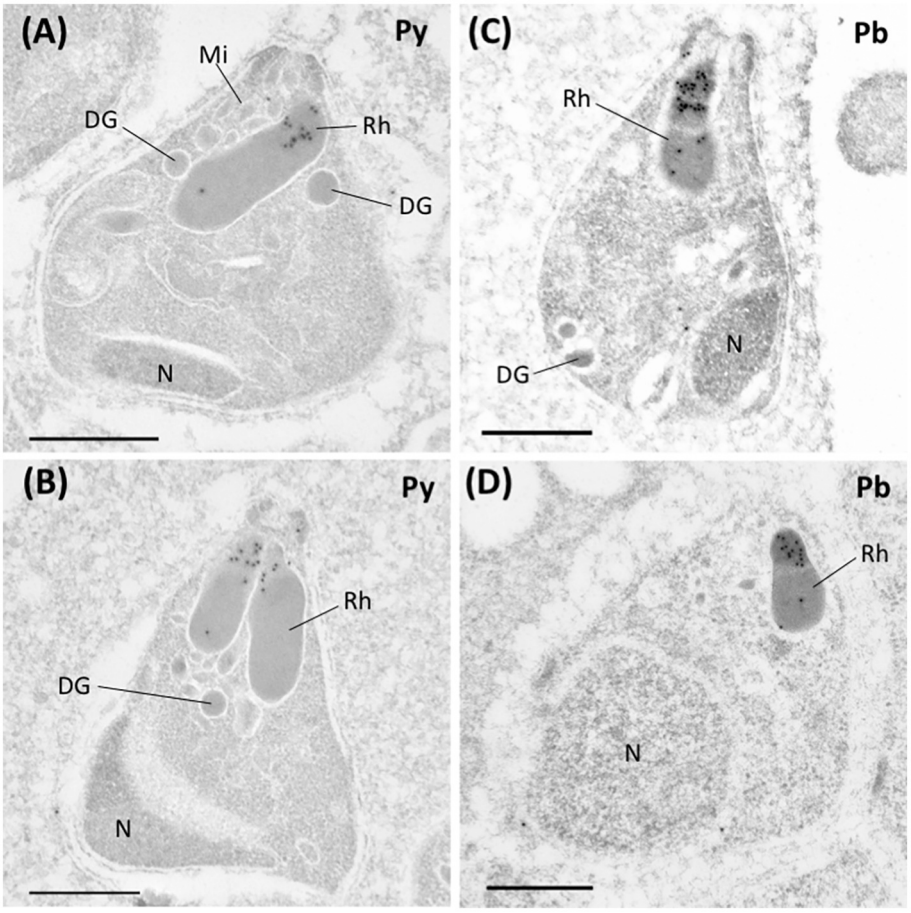

Fig. 2. Subcellular localization of RON12 in merozoite.
(A and B) Immunoelectron microscope images of *P. yoelii* schizonts. Longitudinal sections of the rhoptries are shown. Presence of RON12 is indicated by 15 nm gold particles observed on the neck portion of the rhoptry. (C and D) Immunoelectron microscopy of *P. berghei* schizonts. Longitudinal sections of the rhoptry are shown. Presence of RON12 is indicated by the 15 nm gold particles deposited on the neck region of the rhoptry. Rh: rhoptry. Mi: microneme. DG: dense granule. N: nucleus. Scale bars, 500 nm.

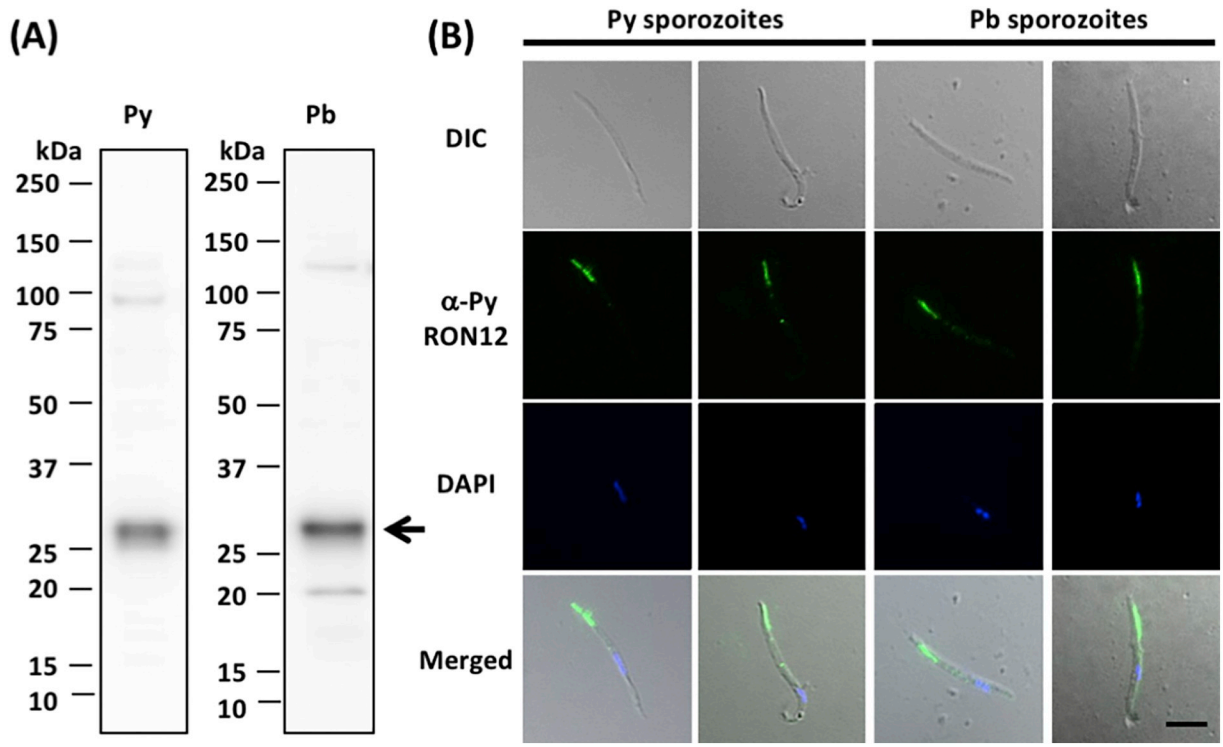

Fig. 3. Expression of RON12 in sporozoites of *P. yoelii* and *P. berghei*.
(A) Western blotting analyses of RON12 in *P. yoelii* and *P. berghei* sporozoites. Parasite lysates of sporozoites collected from midguts from *P. yoelii* or *P. berghei* infected mosquitoes were separated by SDS-PAGE under non-reducing conditions and subjected to immunoblotting with anti-PyRON12 antibodies at 1 μg/ml. Major bands corresponding to PyRON12 and PbRON12 are indicated by an arrow. (B) Expression of RON12 in sporozoites revealed by IFA. RON12 signals were detected in the apical region in both *P. yoelii* and *P. berghei* sporozoites using anti-PyRON12 antibodies at 2 μg/ml. Nuclei were stained with DAPI (blue). Scale bar, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

punctate RON12 signal is additionally detected at the marginal region of parasites as well as the central area, but remain within the parasite rather than being secreted to the PV space. It is noteworthy that this RON12 localization pattern is similar to that in ring stage parasites [22]. The signal intensity of RON12 increases during intra-hepatocytic development, suggesting that newly RON12 production occurs during liver stage parasite maturation.

Detailed localization analysis of rhoptry proteins in merozoites revealed that molecules important for merozoite invasion of erythrocytes, such as the RON complex, localize to the neck portion of rhoptries [2,9]. In contrast, profiling of rhoptry proteins in sporozoites is still limited, and only the expression of RON2, RON4, and RON5 in sporozoites is confirmed by IFA. Among these RON proteins, *P. yoelii* RON5 is observed to be localized to the rhoptry body by IEM; albeit with incomplete analysis of the rhoptry neck [18]. Here, we clearly demonstrated by IEM that RON12 is expressed in sporozoites and localizes to the rhoptry body rather than the neck in both *P. yoelii* and *P. berghei*. These findings suggest that localization of RON proteins within rhoptries can differ between merozoites and sporozoites in *Plasmodium*. The finding that AMA1, which plays an important role in merozoite

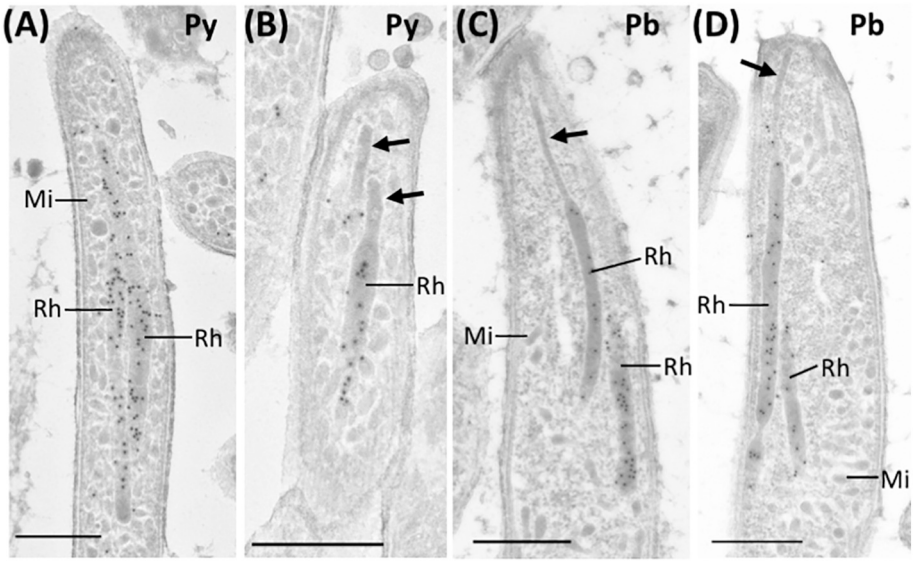

Fig. 4. Subcellular localization of RON12 in sporozoites.
P. yoelii sporozoites in salivary glands at day 16 (A and B) and *P. berghei* sporozoites in salivary glands at day 24 (C and D) were observed by immunoelectron microscopy. RON12 localization is indicated by 15 nm gold particles observed in the body region of rhoptries, but not the neck portion (indicated by arrows). Rh: rhoptry. Mi: microneme. Scale bars, 500 nm.

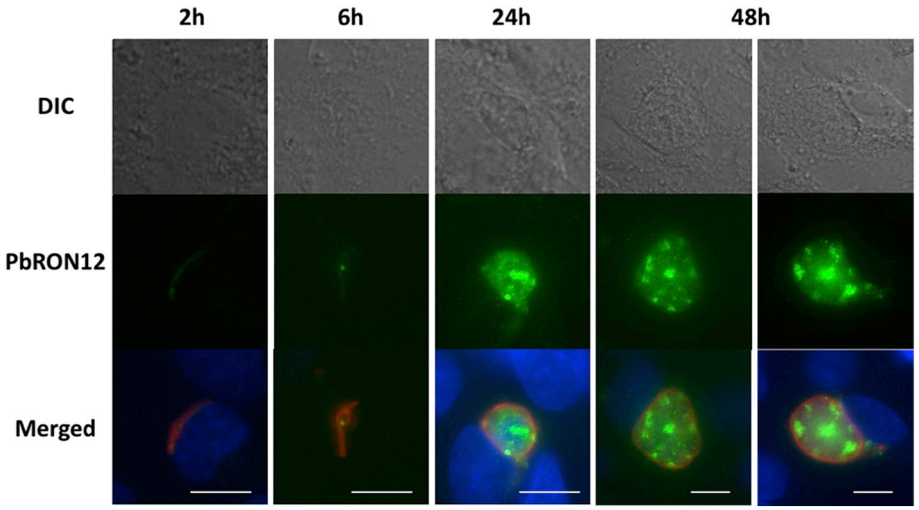

Fig. 5. Subcellular localization of RON12 in liver stage parasites.
Intra-hepatocytic sporozoite localization of RON12 at 2 h, 6 h, 24 h, and 48 h after inoculation of HepG2 cells with Pb sporozoites collected from salivary glands of infected mosquitoes at 19 days post feeding. HepG2 cells were fixed with 10% formalin and permeabilized with methanol. Sporozoites and liver stage parasites are stained with anti-CSP monoclonal antibodies (red). RON12 (indicated in green) mostly remains in the cytoplasm of parasites in the liver stage parasites as a dotted pattern. At the late liver schizont stage, RON12 is detected as spots at the marginal region of the parasites. Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

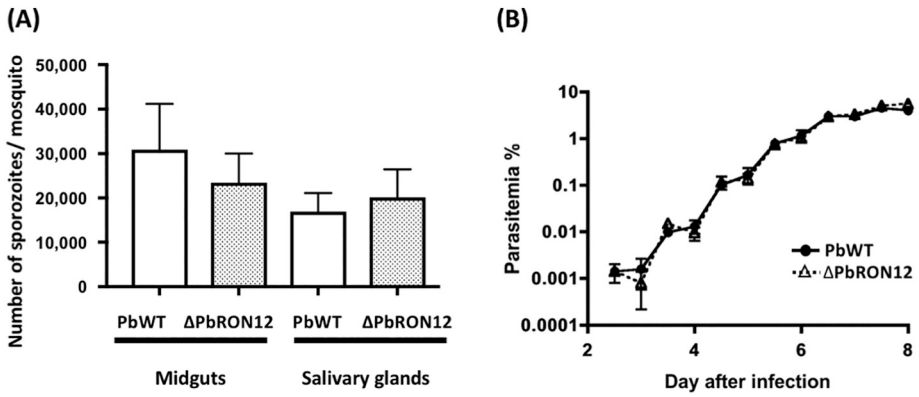

Fig. 6. Phenotypic analysis of ΔPbRON12 during mosquito and liver stage development.
(A) Comparison of sporozoite numbers collected from midguts or salivary glands from PbWT or ΔPbRON12 infected mosquitoes. At least 28 infected mosquitoes were dissected to count the numbers of sporozoites in each parasite line at day 21–23 post-feeding. Bars indicate the mean numbers of sporozoites with standard errors from four independent experiments. There was no significant difference between PbWT and ΔPbRON12 in sporozoite numbers in either the midguts or salivary glands. The statistical analysis was performed by Mann-Whitney *U* test. (B) Representative data showing changes in peripheral blood parasitemias of infected mice after injection of 30,000 PbWT (closed circle) or ΔPbRON12 (open triangle) sporozoites intravenously. Infected erythrocytes appeared 2 days after sporozoite administration in both groups, and no significant difference was observed in parasitemias.

invasion of erythrocytes by binding to the RON complex, is not essential for sporozoite infection of hepatocytes in *P. berghei* [17,33] suggests that rhoptry proteins may have different roles in merozoites and sporozoites. Given the possibility that differences in the localization of the molecules within the rhoptry may reflect functional differences, it is desirable to compare the detailed localization of other RON proteins in merozoites and sporozoites by IEM, particularly RON2 and RON4, which play important roles in erythrocyte invasion.

3.3. RON12 is dispensable for salivary gland invasion and liver infection

It is reported that numerous rhoptry proteins of *Plasmodium* are essential for erythrocytic stage parasite survival [16,17,34], whereas RON12 is not essential for intraerythrocytic stage parasite development [22]. We investigated whether RON12 is necessary for sporozoite migration within mosquito bodies, in particular to invade salivary glands. For this, we generated ΔPbRON12 by double homologous recombination in *P. berghei* (Fig. S2A). The *pbron12* gene was successfully replaced with a drug resistant cassette, as indicated by genotyping PCR (Fig. S2B). The absence of RON12 protein expression in both schizonts and sporozoites of ΔPbRON12 was confirmed by lack of reactivity with anti-PyRON12 antibodies on Western blotting (Fig. S2C). Since ΔPbRON12 could proliferate in ICR mice with parasitemias similar to PbWT, groups of *A. stephensi* mosquitoes were fed to ΔPbRON12 or PbWT infected mice, respectively. The numbers of sporozoites formed within mosquito midgut oocysts were not significantly different between PbWT and ΔPbRON12 (Fig. 6A). Also, there was no difference between ΔPbRON12 and PbWT in the number of sporozoites recovered from salivary glands (Fig. 6A). These results demonstrate that ΔPbRON12 sporozoites were viable and capable of multiplying in oocysts and invading salivary glands in mosquitoes at similar levels as wild type. The report that RON4 plays an important role during sporozoite infection of hepatocytes [17] raised the question whether RON12 is also involved in sporozoite infection of hepatocytes. Sporozoites collected from salivary glands of mosquitoes infected with ΔPbRON12 or PbWT were intravenously injected into ICR mice. Three independent infections with five mice for each parasite line were performed. The patency of parasite appearance in the peripheral blood of infected mice was monitored every 12 h from day 2 to day 8. Parasitemias were detected on day 2 of infection, then increased and reached 10% on day 8. No significant difference in parasitemia was observed between ΔPbRON12 infected mice and PbWT infected mice (Figs. 6B, S3). These results indicate that ΔPbRON12 sporozoites invade hepatocytes and differentiate into infective hepatic merozoites with the same efficiency as PbWT; and, as a result, ΔPbRON12 and PbWT parasites appear simultaneously in the peripheral blood. Therefore, it is concluded that PbRON12 is neither essential for blood-stage growth nor sporozoite invasion and development in the liver.

Because a phenotype was not observed in our assays on *pbron12*-disrupted parasites, we could not describe a function of RON12 in hepatic development, noting here the thus far anonymous role of RON12 during erythrocytic invasion and development. However, since RON12 orthologs are found across *Plasmodium*, and with high amino acid sequence conservation, RON12 is likely to have a conserved function during invasion of target cells, albeit dispensable or subject to compensation. As RON12 is localized to the marginal region of parasites in

erythrocytes and hepatocytes, it is also possible that RON12 has some, redundant, roles for parasite maturation in host cells. Further work on the identification of RON12 interacting proteins in sporozoites, as well as in hepatocytes may help elucidate the role of RON12 in sporozoites. In addition, functional analyses of rhoptry proteins in sporozoites using a conditional knockdown system will give clues regarding the comprehensive molecular mechanisms of sporozoite invasion of target cells.

4. Conclusions

The RON complex consisting of RON2, RON4, and RON5 plays an important role in the formation of the moving junction during merozoite invasion of erythrocytes. In contrast, little is known regarding the function of rhoptry proteins in sporozoites, the invasive forms of mosquito salivary glands and mammalian host hepatocytes. In this study, we demonstrated that RON12 is expressed in both merozoites and sporozoites; however, its localization in rhoptries differs between the two parasite stages. Specifically, RON12 is restricted to the rhoptry neck in merozoites whereas it is distributed through the rhoptry body in sporozoites. Phenotypic analysis of pb*ron12*-disrupted parasites showed that RON12 is dispensable for sporogony, sporozoite invasion of salivary gland and hepatocytes, and development in hepatocytes; and thus its function remains anonymous in both *Plasmodium* merozoite and sporozoite stages.

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Conflicts of interest statement

The authors are not aware of any conflicts of interest arising from this work.

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