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Plasmodial ortholog of *Toxoplasma gondii* rhoptry neck protein 3 is localized to the rhoptry body

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ABSTRACT

The proteins in apical organelles of *Plasmodium falciparum* merozoite play an important role in invasion into erythrocytes. Several rhoptry neck (RON) proteins have been identified in rhoptry proteome of the closely-related apicomplexan parasite, *Toxoplasma gondii*. Recently, three of *P. falciparum* proteins orthologous to *TgRON* proteins, *Pf*RON2, 4 and 5, were found to be located in the rhoptry neck and interact with the micronemal protein apical membrane antigen 1 (*Pf*AMA1) to form a moving junction complex that helps the invasion of merozoite into erythrocyte. However, the other *P. falciparum* RON proteins have yet to be characterized. Here, we determined that "PFL2505c" (hereafter referred to as *pfron3*) is the ortholog of the *tgron3* in *P. falciparum* and characterized its protein expression profile, subcellular localization, and complex formation. Protein expression analysis revealed that *Pf*RON3 was expressed primarily in late schizont stage parasites. Immunofluorescence microscopy (IFA) showed that *Pf*RON3 localizes in the apical region of *P. falciparum* merozoites. Results from immunoelectron microscopy, along with IFA, clarified that *Pf*RON3 localizes in the rhoptry body and not in the rhoptry neck. Even after erythrocyte invasion, *Pf*RON3 was still detectable at the parasite ring stage in the parasitophorous vacuole. Moreover, co-immunoprecipitation studies indicated that *Pf*RON3 interacts with *Pf*RON2 and *Pf*RON4, but not with *Pf*AMA1. These results suggest that *Pf*RON3 partakes in the novel *Pf*RON complex formation (*Pf*RON2, 3, and 4), but not in the moving junction complex (*Pf*RON2, 4, 5, and *Pf*AMA1). The novel *Pf*RON complex, as well as the moving junction complex, might play a fundamental role in erythrocyte invasion by merozoite stage parasites.

1. Introduction

Malaria is caused by the replication of protozoan parasites of the genus *Plasmodium* in circulating host erythrocytes [1]. The invasion process of merozoite stage parasite into erythrocyte requires the discharge of contents of apical secretory organelles (micronemes and rhoptries) to form an irreversible contact, called a tight junction, between the merozoite and the erythrocyte membrane. This tight junction migrates from the anterior to posterior poles of the merozoite. According to this moving junction, the host membrane invaginates the merozoites to eventually form a parasitophorous vacuole [2,3]. The moving junction is one of the most distinctive features of apicomplexan invasion into host cells, and was first observed in *Plasmodium* species [4]. Studies in apicomplexan parasite *Toxoplasma gondii* identified a total of four proteins from distinct apical secretory organelles to form a moving junction complex; micronemal protein apical membrane antigen 1 (AMA1) and three rhoptry neck (RON) proteins, RON2, RON4, and RON5 [5,6]. Recently, this RON–AMA1 complex (*Pf*RON2, *Pf*RON4, *Pf*RON5, and *Pf*AMA1) was also identified in *Plasmodium falciparum* [7–9]. Attempts to knockout the AMA1 gene locus were unsuccessful in both *Plasmodium* [10] and *T. gondii* [11]. AMA1-binding peptide R1 not only prevents RON–AMA1 complex interaction, but also blocks *P. falciparum* merozoite invasion into erythrocytes [9].

Although the cumulative evidence above strongly suggests that the conserved RON–AMA1 complex plays an essential role in merozoite invasion, it is yet to be clarified whether molecules other than RON2, RON 4, and RON 5, play roles in the formation of the moving junction complex and in the invasion process. The report on *T. gondii* rhoptry proteome identified the presence of other RON proteins, RON1 and RON3 [12]. Therefore, we were interested in

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identifying the ortholog of *tgron3* in *P. falciparum* and in characterizing its protein expression profile, subcellular localization, and role in the formation of the RON–AMA1 complex.

2. Materials and methods

2.1. Parasites

P. falciparum asexual stages (3D7 strain) were maintained in human erythrocytes (blood group O^+) *in vitro*, as previously described [13].

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *P. falciparum* schizont-infected erythrocytes (purified by differential centrifugation on a 70%/40% Percoll/sorbitol gradient) using the TRizol Reagent (Invitrogen, Carlsbad, CA). Following DNase treatment, cDNA was generated with a random hexamer using the SuperScriptIII® First Strand Synthesis System (Invitrogen).

2.3. Antibodies

Recombinant *Pf*RON3 proteins were produced using the wheat germ cell-free translation system (CellFree Sciences, Matsuyama, Japan) as described previously [14–16]. Briefly, regions of the *Pf*RON3 gene encoding the deduced amino acid sequence, 927–1056 (*Pf*RON3_1) and 1686–1884 (*Pf*RON3_2), were PCR-amplified from *P. falciparum* 3D7 blood-stage cDNA and ligated into pEU-E01-GST-(TEV)-N2 (CellFree Sciences), an expression plasmid with an N-terminal glutathione S transferase (GST)-tag followed by a tobacco etch virus (TEV) protease cleavage site, designed specifically for the wheat germ cell-free protein expression. Oligonucleotide primers used in the PCR amplification were *Pf*RON3_XhoIF1 (5′-ctcgagGATATTCCATTAAAAGAAACCTATAAATT-3′) and *Pf*RON3_BamHIR1 (5′-ggatccCTAATGTGGGAACATTTCATGATTTGGTA-3′) for *Pf*RON3_1, and *Pf*RON3_XhoIF2 (5′-ctcgagGATTTTAAAGATAAATCAGATGATGATC-3′) and *Pf*RON3_BamHIR2 (5′-ggatccCTATTTTTTAGGTACATATATATTATATGGTC-3′) for *Pf*RON3_2 (XhoI and BamHI restriction sites are underlined). Both GST-*Pf*RON3_1 and GST-*Pf*RON3_2 were captured using a glutathione-Sepharose 4B column (GE Healthcare, Camarillo, CA), and eluted by on-column cleavage with 60 U of AcTEV protease (Invitrogen) after extensive washing with PBS. To generate anti-*Pf*RON3_1 and anti-*Pf*RON3_2 sera, female BALB/c mice were immunized subcutaneously with 20 μg of purified *Pf*RON3_1 or *Pf*RON3_2 emulsified with Freund's adjuvant. A Japanese white rabbit was also immunized subcutaneously with 250 μg of purified *Pf*RON3_1 or *Pf*RON3_2 emulsified with Freund's adjuvant. All immunizations were performed 3 times at 3-week intervals, and then antisera were collected 2 weeks after the third immunization. In a similar manner, mouse anti-*Pf*RAP1 (aa 1–782) antibody, mouse anti-*Pf*EXP2 (aa 25–287) antibody, mouse and rabbit anti-*Pf*AMA1 (aa 25–546) antibodies, and rabbit anti-GST antibody, were generated as control. Rabbit antisera against the *Pf*RON3_1 and *Pf*RON3_2 proteins were affinity purified using a column conjugated with recombinant *Pf*RON3_1 or *Pf*RON3_2 as ligands, respectively. Briefly, recombinant *Pf*RON3_1 or *Pf*RON3_2 was covalently linked to a HiTrap™ NHS-activated HP column (GE Healthcare) as manufacturer's recommendation. Rabbit antiserum was then applied to either the *Pf*RON3_1- or the *Pf*RON3_2-conjugated column. After an extensive washing step with 20 mM phosphate buffer (pH 7.0), antigen-specific IgGs were eluted with 0.1 M glycine–HCl (pH 2.5), and then immediately neutralized with 1 M Tris (pH 9.0). Mouse monoclonal anti-*Pf*RON4 antibody (26C64F12) [7] and anti-*Pf*RESA antibody (23/9) [17] were kind gifts from Jean F. Dubremetz (Université de Montpellier 2, France) and Robin F. Anders (La Trobe University, Australia), respectively.

2.4. SDS-PAGE and western blot analysis

P. falciparum cultured parasites were harvested after tetanolysin (List Biological Laboratories, Inc., Campbell, CA) treatment that can remove the hemoglobin without loss of parasite proteins present in the parasitophorous vacuolar space [18]. The parasite proteins were then extracted in SDS-PAGE loading buffer, incubated at 4 °C for 6 h, and subjected to electrophoresis under reducing condition on a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were then transferred to a 0.2-μm PVDF membrane (GE Healthcare). The proteins were immunostained with antisera followed by horseradish peroxidase-conjugated secondary antibody (GE Healthcare) and visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on a LAS 4000 mini luminescent image analyzer (GE Healthcare). The relative molecular masses of the proteins were estimated with reference to Precision Plus Protein Standards (BioRad, Hercules, CA).

2.5. Immunoprecipitation

Immunoprecipitation was carried out as previously described [19]. Briefly, proteins were extracted from late schizont parasite pellets in PBS with 1% Triton X-100 containing Complete Proteinase Inhibitor Cocktail (Roche, Indianapolis, IN). Supernatants (50 μl) were pre-incubated at 4 °C for 1 h with 40 μl of 50% protein G-conjugated beads (GammaBind Plus Sepharose; GE Healthcare) in NETT buffer (50 mM Tris–HCl, 0.15 M NaCl, 1 mM EDTA, and 0.5% Triton X-100) supplemented with 0.5% BSA (fraction V; Sigma-Aldrich Corporation, St. Louis, MO). Aliquots of recovered supernatants were incubated either with rabbit anti-*Pf*RON3_1, anti-*Pf*AMA1, anti-*Pf*RON2, or anti-GST antibody, and then 40 μl of 50% protein G-conjugated bead suspension was added. After one-hour incubation at 4 °C, the beads were washed once with NETT–0.5% BSA, once with NETT, once with high-salt NETT (0.5 M NaCl), once with NETT, and once with low-salt NETT (0.05 M NaCl and 0.17% Triton X-100). Finally, proteins were eluted from the protein G-conjugated beads with 0.1 M glycine–HCl (pH 2.5), and then immediately neutralized with 1 M Tris pH 9.0. The supernatants were used for western blot analysis.

2.6. Indirect immunofluorescence assay

Thin smears of ring or schizont-enriched *P. falciparum*-infected erythrocytes were prepared on glass slides and stored at −80 °C. The smears were thawed, fixed with 4% formaldehyde at room temperature for 10 min, permeabilized with PBS containing 0.1% Triton X-100 at room temperature for 15 min, and blocked with PBS containing 5% non-fat milk at 37 °C for 30 min. The smears were then incubated with rabbit anti-*Pf*RON3 antibodies (1:500 dilution) and control mouse antibodies at 37 °C for 1 h, followed by incubation with both Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG (Invitrogen) as secondary antibodies (1:500) at 37 °C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (2 μg/ml, DAPI) mixed with a secondary antibody solution. Slides were mounted in ProLong Gold Antifade reagent (Invitrogen) and viewed under ×63 oil-immersion lens. High-resolution image-capture and processing were performed using a confocal scanning laser microscope (LSM5 PASCAL or LSM710; Carl Zeiss MicroImaging, Thornwood, NY). Images were processed in Adobe Photoshop (Adobe Systems Inc., San José, CA).

2.7. Immunoelectron microscopy

Parasites were fixed for 15 min on ice in a mixture of 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed specimens were washed, dehydrated, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) as previously

have been described [20,21]. Ultrathin sections were blocked in PBS containing 5% non-fat milk and 0.01% Tween 20 (PBS-MT) at 37 °C for 30 min. Grids were then incubated at 4 °C overnight with rabbit anti-*Pf*RON3_2 or control sera in PBS-MT (1:20). After washing with PBS containing 10% BlockAce (Yukijirushi, Sapporo, 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 10 nm gold particles (GE Healthcare) diluted 1:20 in PBS-MT, rinsed with PBS-BT, and fixed at room temperature for 10 min in 2.5% glutaraldehyde to stabilize the gold particles. The grids were then rinsed with distilled water, dried, and stained with uranyl acetate and lead citrate. Samples were examined with a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan).

3. Results

3.1. Primary structure analysis of the RON3 orthologs

Using *Tg*RON3 (TGME49_023920) as a query in BLAST analyses [22], we found RON3 orthologs in *P. falciparum* (*Pf*RON3; PFL2505c, PlasmoDB) [23], *P. yoelii* 17XNL strain (*Py*RON3; PY01808, PlasmoDB), *P. vivax* Sal-I strain (*Pv*RON3; PVX_101485, PlasmoDB), *P. knowlesi* H strain (*Pk*RON3; PKH_146960, PlasmoDB), *P. chabaudi* AS strain (*Pch*RON3; PCA_146710, PlasmoDB), *P. berghei* ANKA strain (*Pb*RON3; PBANKA_146490, PlasmoDB), and *Eimeria tenella* (*Et*RON3; CAO79912, Sanger Institute). The full-length *Pf*RON3 protein consists of 2215 amino acid residues, with a putative N-terminal signal peptide sequence predicted by SignslP3.0 [24] to span amino acid residues 1 – 22. Three transmembrane regions (TM) (aa 250–272, 276–298, and 551–573) were predicted by TMHMM2.0 [25], and one coiled coil region (aa 1822–1847) was predicted by SMART [26]. Alignment of the deduced amino acid sequences of all the RON3 orthologs among the genus *Plasmodium* using Clustal W [27] demonstrated that twelve Cys residues are conserved (Fig. 1), with a 42% overall sequence identity. Moreover, the N-terminal region is highly conserved among the RON3 orthologs in the genus *Plasmodium*, in *T. gondii*, and in *E. tenella* (Fig. 2).

3.2. PfRON3 is expressed in the schizont stage and existed in the ring stage

The PfRON3 protein expression profile was analyzed by stage-specific western blot analysis using tightly synchronized parasites as antigens. Both antibodies (α-*Pf*RON3_1 and _2) recognized a band slightly larger than 250 kDa corresponding to the predicted molecular mass of *Pf*RON3 in late stage parasites, 40–44 h post invasion (Fig. 3A, arrowhead). Furthermore, both antibodies also recognized a prominent 190-kDa band, 40–44 h post invasion (Fig. 3A, arrow). After erythrocyte invasion, the 190-kDa band quickly degraded to 50- and 40-kDa bands (Figs. 3A and 4H). However, a small amount of the 190-kDa band was present throughout the ring (Figs. 3A and 4H) and trophozoite stages (Fig. 3A, 24–36 h). Additionally, only the anti-*Pf*RON3_2 antibody recognized a 37-kDa band, 40–44 h post invasion that might represent one of the processed forms of the *Pf*RON3 protein (Fig. 3A). Neither anti-PfRON3_1 or -PfRON3_2 had any reactivity against antigens of uninfected erythrocytes (Supplementary Fig. S1).

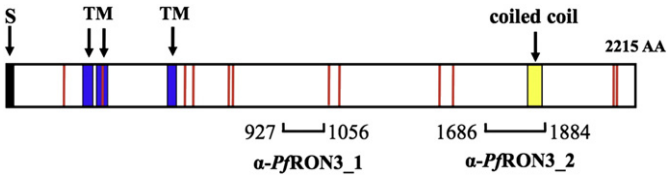

Fig. 1. Schematic representation of *Pf*RON3. The primary structure of *Pf*RON3 is depicted based on the analyses described in the Results section. S and TM indicate putative signal peptide (black) and transmembrane (blue) sequences, respectively. The yellow color box indicates a coiled coil region. Vertical red bars indicate conserved Cys residues in orthologs among the genus *Plasmodium*. The regions used to generate anti-*Pf*RON3 sera (α-*Pf*RON3_1 and α-*Pf*RON3_2) are indicated.

3.3. PfRON3 localizes in the rhoptry body of merozoites

In order to determine the sub cellular localization of *Pf*RON3, a dual label indirect immunofluorescence assay (IFA) was performed using anti-*Pf*RON3_2 antibody with anti-*Pf*RAP1 (rhoptry body marker), anti-*Pf*RON2 (rhoptry neck marker), anti-*Pf*AMA1 (microneme marker), and anti-*Pf*RESA (dense granule marker) antibodies as controls (Fig. 4). In mature schizonts, the anti-*Pf*RON3 antibody produced a punctate pattern of fluorescence in the apical end of each developing merozoite. Although some of the *Pf*RON3 signals overlapped with those of *Pf*RON2, *Pf*AMA1, or *Pf*RESA the *Pf*RON3 signals did not completely colocalize with those of the controls, whereas complete colocalization was observed between the signals of PfRON3 and *Pf*RAP1. Negative control sera were always used and these images were found to be negative (data not shown). To confirm the IFA results, the precise subcellular localization of *Pf*RON3 in the merozoite. Using anti-*Pf*RON3 antibody, gold particle signals were detected in the body portion of the rhoptries (Fig. 5).

3.4. PfRON3 is found in the parasitophorous vacuolar space after merozoite invasion into erythrocyte

To investigate *Pf*RON3 localization after the parasite invades erythrocytes, we performed IFA on ring stage parasites. *Pf*RAP1 and *Pf*EXP2 were used as parasitophorous vacuolar (PV) markers because they have been demonstrated to be present in ring stages and to associate with the PV [28–30]. We found that *Pf*RON3 colocalized with the PV markers, *Pf*RAP1 and *Pf*EXP2 in a discrete compartment surrounding the ring stage parasites (Fig. 6A). Next we performed western blot analysis using extracts of tightly synchronized ring or schizont stage parasites (Fig. 6B). *Pf*RON3 was detected in ring and schizont stage parasites together with *Pf*RAP1 and *Pf*EXP2. However *Pf*RON2 and *Pf*RON4 were detected only in schizont stage parasites (Fig. 6B) in agreement with the IFA results (Fig. 6A). Negative control sera were always used and these images were found to be negative (data not shown).

3.5. PfRON3 forms a complex with PfRON2 and PfRON4, but not with PfAMA1

To evaluate the interaction between *Pf*RON3 and the RON–AMA1 complex, we performed immunoprecipitation experiments using mature schizont-rich parasite extracts (Fig. 7). We did not detect *Pf*RON3 in the immunoprecipitate obtained using anti-*Pf*AMA1 antibody. However, PfRON3 was found in the anti-*Pf*RON2 precipitates. In the reciprocal experiment, *Pf*RON2 and *Pf*RON4 were detected in the immunoprecipitate obtained using the anti-*Pf*RON3_1 antibody; however, *Pf*AMA1 was not detected (Fig. 7). There was negligible crossreactivity between the anti-PfRON3_1 or anti-PfRON3_2 IgGs and PfRON2 and PfRON4 (Supplementary Fig. S1). These faint crossreactions were insignificant compared to the reactions with the actual target molecules.

4. Discussion

In this study, we characterized the protein expression profile and subcellular localization of *P. falciparum* RON3 as well as its complex formation with *Pf*RON2 and *Pf*RON4.

Studies of the apicomplexan parasite *T. gondii* identified various *Tg*RON proteins (RON2, RON4, RON5 [5,6], RON1, and RON3 [12]). The potential orthologs of the *Tg*RON proteins were subsequently

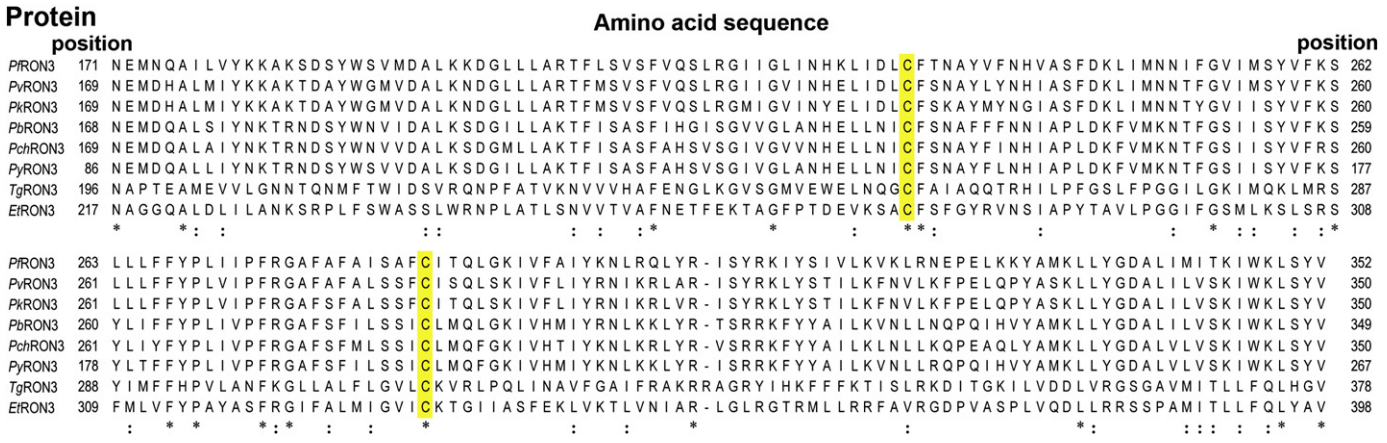

Fig. 2. Amino acid alignment of the conserved N-terminal region of RON3 orthologs. Deduced amino acid sequences of RON3 orthologs were aligned using Clustal W with manual correction. "*" indicates the conserved residues in the aligned sequences. ":" indicates conservative substitutions. Cys residues are highlighted in yellow.

identified in *P. falciparum*. Bradley et al. [12] suggested that *Pf*RON3 (PFL2505c) is an ortholog of *Tg*RON3 (TGME49_023920). However, Proellocks et al. [31] analyzed the amino acid sequence similarities of the RON orthologs identified in the genera *Plasmodium*, *Toxoplasma*, and some (but not all) *Apicomplexa*, and reported that, they may not be true orthologs because the collective sequence identity is below 12% [31]. In contrast, our pair-wise amino acid alignments between *T. gondii* and *P. falciparum* RON orthologs showed 11% identity between RON1 orthologs (TGME49_110010 vs. PFD0207c), 14% between RON2 orthologs(TGME49_100100 vs. PF14_0495), 12% between RON3 orthologs (TGME49_023920 vs. PFL2505c), and 10% between RON4 orthologs (TGME49_029010 vs. PF11_0168), suggesting that the amino acid identity between RON3 orthologs (*Tg*RON3 and *Pf*RON3) is comparable to that of other RON orthologs. Furthermore, multiple alignments of the deduced amino acid sequences of apicomplexan parasite RON3 orthologs showed that the N-terminal regions are conserved (Fig. 2). Based on these analyses, we conclude that *Pf*RON3 (PFL2505c) is an authentic ortholog of *Tg*RON3 (TGME49_023920).

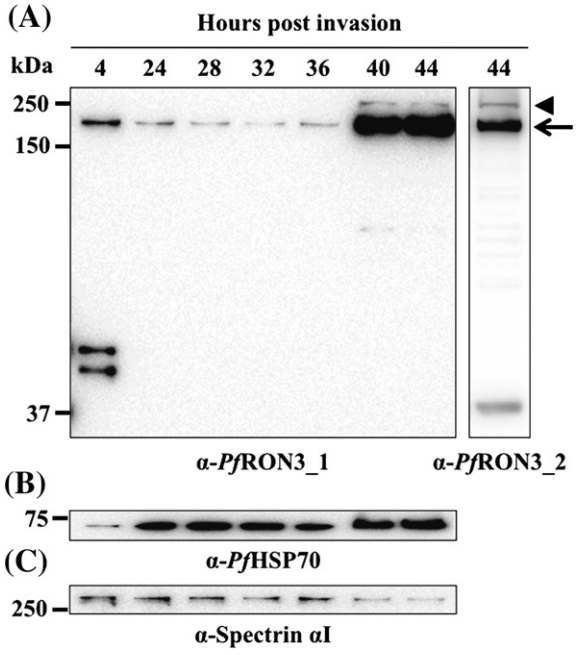

Fig. 3. Stage-specific expression profile of *Pf*RON3. Proteins from synchronized parasite cultures were harvested at each time point and separated by SDS-PAGE on a 12.5% gel under reducing condition. (A) Using either anti-*Pf*RON3_1- or anti-*Pf*RON3_2- specific antibodies, a band of approximately 260 kDa (arrowhead) was detected in late stages (40–44 h post invasion) and a 190-kDa band (arrow) was detected in both the schizont, ring, and trophozoite stage parasites. (B and C) Loading controls. To ensure that equal amounts of the stage-specific samples were loaded in each lane for western blot analysis, the membranes were probed with anti-*Pf*HSP70 monoclonal antibody (4C9) as a quantitative parasite protein marker [36], and anti-human spectrin α I rabbit antibody indicating the number of erythrocytes. The intensity of the *Pf*HSP70 bands indicated that the amount of sample loaded in each lane was comparable.

T. gondii RON3 protein was originally suggested to be localized at the rhoptry neck by immunofluorescence assay [12]. In contrast, our current immunoelectron microscopy results confirmed that *Pf*RON3 localizes in the rhoptry body rather than the rhoptry neck. Since *Pf*RON3 is in the rhoptry body, we are tempted to think that TgRON3 is also localizes in the rhoptry body rather than the rhoptry neck. Therefore we believe that the localization of *Tg*RON3 should be reconfirmed by electron microscopy using quality antibodies. In general, the biggest technical hurdle to obtaining antibodies of adequate quality for use in electron microscopy studies of this sort would be the ability to produce correctly folded recombinant proteins in sufficient quantity and purity [31]. The immunoelectron microscopy results we obtained clearly demonstrate that high quality antibodies can be successfully produced using quality proteins synthesized in the wheat germ cell-free protein production system. We have already [14–16] demonstrated that the wheat germ cell-free protein production system enables the expression of quality recombinant proteins without codon optimization.

In our stage-specific western blot and immunofluorescence microscopy analyses for the *Pf*RON3 expression profile, we detected *Pf*RON3 not only in the merozoite rhoptry body but also in the PV of ring stage parasites (Fig. 6). Previous studies showed that well-characterized rhoptry body proteins (*Pf*RhopH complex, *Pf*RAP complex, and *Pf*RAMA) are discharged into the PV [32–34]. Therefore, the secretory pathway of *Pf*RON3 into the PV might be similar to that of these rhoptry body proteins. Additionally, since the 260-kDa band, corresponding to full-length *Pf*RON3, is only visible at the schizont stage (40–44 h post invasion) (Fig. 3A), the processed 190-kDa fragment may contain the functional domains throughout the parasite's asexual erythrocytic cycle.

Finally, our immunoprecipitation studies demonstrated that *Pf*RON2, *Pf*RON3, and *Pf*RON4, are able to interact and form a novel complex *in vitro*, even in the absence of *Pf*AMA1, indicating that the formation of this novel RON complex is independent of *Pf*AMA1. These results are in agreement with the previously published results showing that *Pf*RON2 and *Pf*RON4 are able to interact and form a complex independently, in the absence of *Pf*AMA1 and in the parasite; most AMA1 is not associated with complexes that contain *Pf*RON2 and *Pf*RON4 [35]. However, in this study we were unable to confirme the

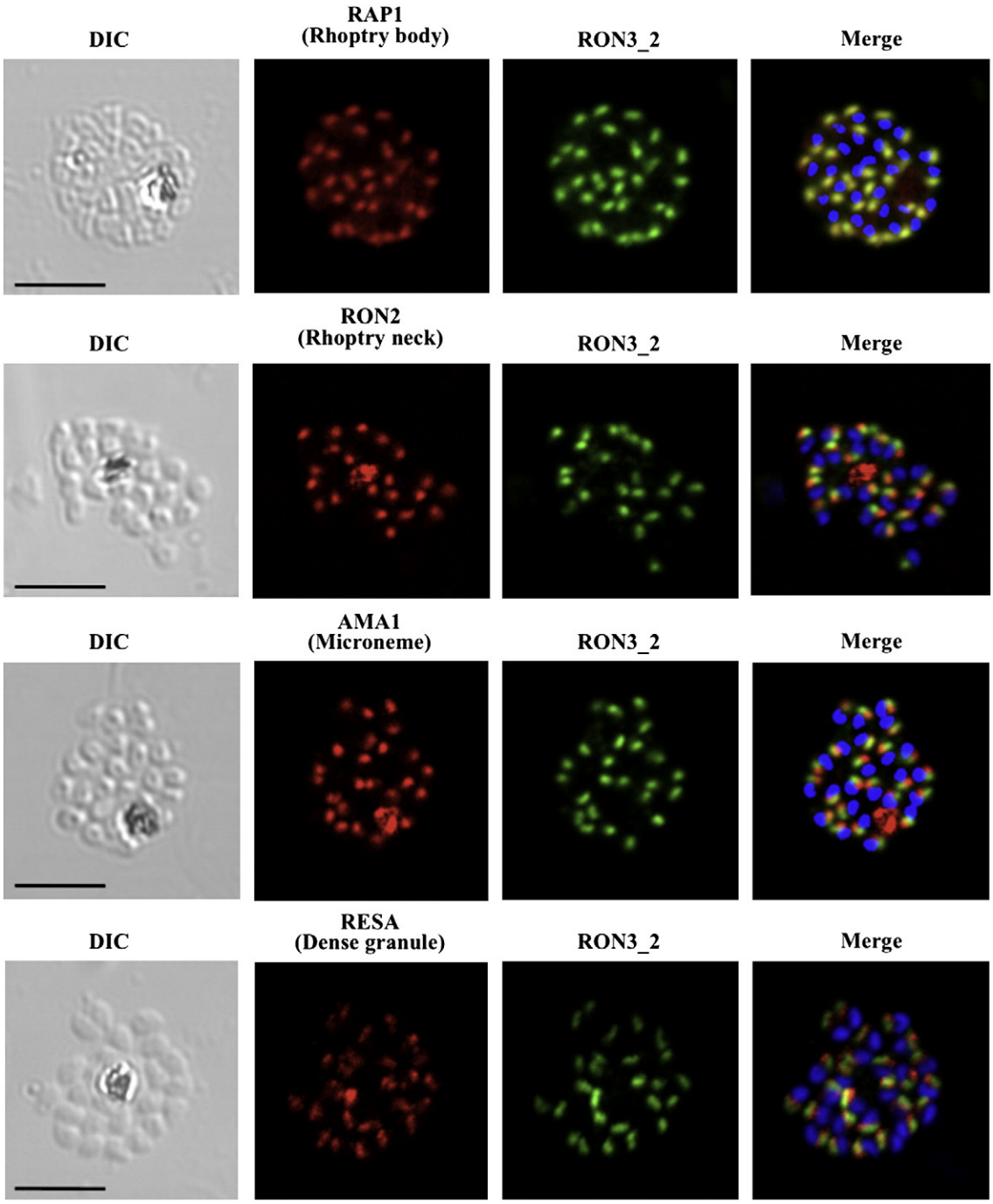

Fig. 4. *Pf*RON3 is expressed at the apical end of *Plasmodium* merozoites. Schizont and merozoite stage parasites were dual-labeled with antisera against *Pf*RON3_2 and either *Pf*RAP1 (rhoptry body marker), *Pf*RON2 (rhoptry neck marker), *Pf*AMA1 (microneme marker), or *Pf*RESA (dense granule marker). Nuclei are visualized with DAPI in merged images shown in the right panels. Bars represent 5 μm.

localization of *Pf*RON3 and the timing of the novel complex formation during the merozoite invasion process. Hence, the role of *Pf*RON3 and the novel complex in formation of the moving junction and the merozoite invasion process could not be elucidated. It will be interesting to undertake further experiments to shed light on the localization and function of *Pf*RON3 and the novel complex during merozoite invasion.

In summary, our results show that *Pf*RON3 is a rhoptry body protein, not a rhoptry neck protein, and that it interacts with *Pf*RON2 and *Pf*RON4, but not with *Pf*AMA1. These results suggest that *Pf*RON3 partakes in the novel *Pf*RON complex formation (*Pf*RON2, 3, and 4), but not in formation of the moving junction complex (*Pf*RON2, 4, 5, and *Pf*AMA1). The novel *Pf*RON complex, as well as the moving junction complex, might play a fundamental role in erythrocyte invasion by merozoite stage parasites.

Acknowledgments

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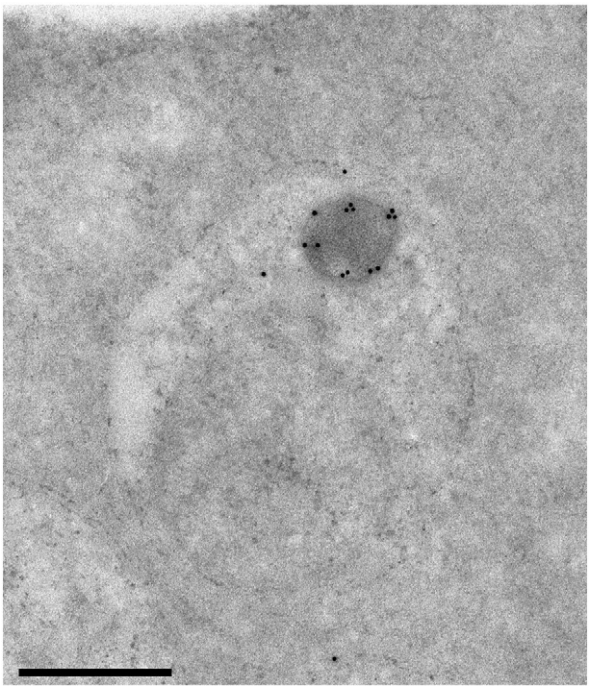

Fig. 5. Rhoptry body localization of *Pf*RON3 by immunoelectron microscopy. Longitudinally sectioned merozoites in mature schizonts were labeled with rabbit anti-*Pf*RON3_2 antibodies followed by secondary antibody conjugated with gold particles. The image shows that the gold particle signals were restricted to the merozoite rhoptry body. Bar represents 500 nm.

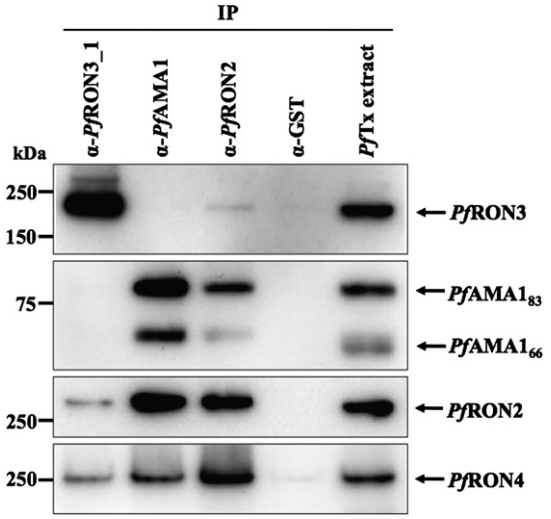

Fig. 7. *Pf*RON3 is not involved in the RON–AMA1 complex. Triton X-100 extracts of schizont-rich parasite (*Pf*Tx extract) were immunoprecipitated (IP) with rabbit sera against *Pf*RON3 (α-*Pf*RON3_1), *Pf*AMA1 (α-*Pf*AMA1), *Pf*RON2 (α-*Pf*RON2), or GST (α-GST), then stained with mouse antisera against *Pf*RON3, *Pf*AMA1, *Pf*RON2, or *Pf*RON4, respectively. Immunoprecipitation using anti-GST antibody was used as a negative control. No bands were detected in the anti-GST immunoprecipitate, indicating the exclusion of potential carryover of proteins due to insufficient or inadequate washing steps.

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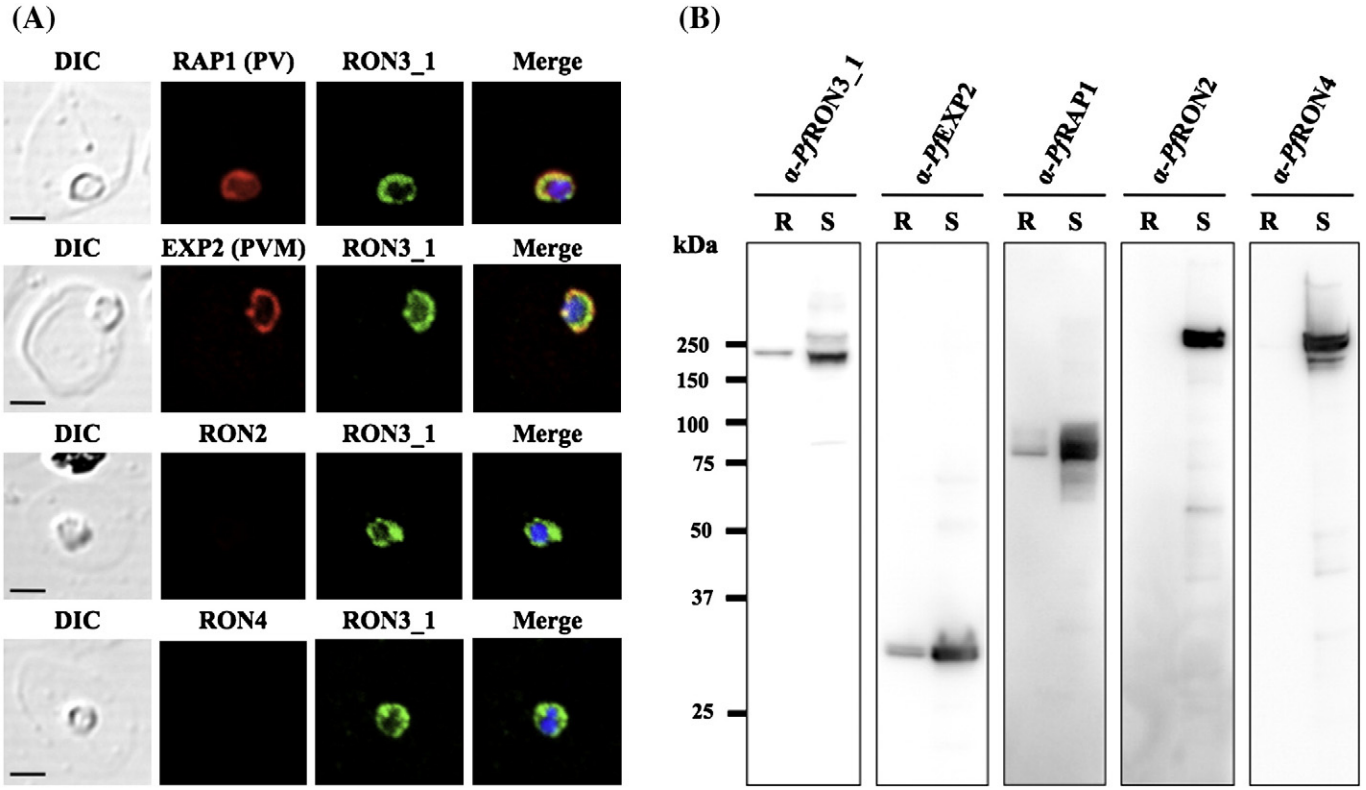

Fig. 6. *Pf*RON3 is found in the parasitophorous vacuole in ring stage parasites. (A) Ring stage parasites were dual-labeled with antisera against *Pf*RON3_1 and either *Pf*RAP1 (PV marker), *Pf*EXP2 (PVM marker), *Pf*RON2, or *Pf*RON4. Nuclei are visualized with DAPI in merged images shown in the right panels. Bars represent 2.5 μm. (B) Proteins from synchronized parasite cultures were harvested at the ring stage (R) and schizont stage (S), and separated by SDS-PAGE on a 12.5% gel under a reducing condition. After transfer of proteins onto a PVDF membrane, the membrane was stained using rabbit anti-*Pf*RON3_1, mouse anti-*Pf*RAP1, anti-*Pf*EXP2, anti-*Pf*RON2, anti-*Pf*RON4 antibodies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.parint.2011.01.001.

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