# 研究成果の刊行に関する一覧表

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雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
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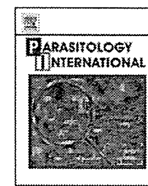

# Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in *Plasmodium falciparum* merozoites ☆

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Rhoptry

A B S T R A C T

Erythrocyte invasion is an essential step in the establishment of host infection by malaria parasites, and is a major target of intervention strategies that attempt to control the disease. Recent proteome analysis of the closely-related apicomplexan parasite, *Toxoplasma gondii*, revealed a panel of novel proteins (RONs) located at the neck portion of the rhoptries. Three of these proteins, RON2, RON4, and RON5 have been shown to form a complex with the microneme protein Apical Membrane Protein 1 (AMA1). This complex, termed the Moving Junction complex, localizes at the interface of the parasite and the host cell during the invasion process. Here we characterized a RON2 ortholog in *Plasmodium falciparum*. *Pf*RON2 transcription peaked at the mature schizont stage and was expressed at the neck portion of the rhoptry in the merozoite. Co-immunoprecipitation of *Pf*RON2, *Pf*RON4 and *Pf*AMA1 indicated that the complex formation is conserved between *T. gondii* and *P. falciparum*, suggesting that co-operative function of the rhoptry and microneme proteins is a common mechanism in apicomplexan parasites during host cell invasion. *Pf*RON2 possesses a region displaying homology with the rhoptry body protein *Pf*RhopH1/Clag, a component of the RhopH complex. However, here we present co-immunoprecipitation studies which suggest that *Pf*RON2 is not a component of the RhopH complex and has an independent role. Nucleotide polymorphism analysis suggested that *Pf*RON2 was under diversifying selective pressure. This evidence suggests that RON2 appears to have a fundamental role in host cell invasion by apicomplexan parasites, and is a potential target for malaria intervention strategies.

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## 1. Introduction

Malaria is one of the most prevalent and deadly global infectious diseases, more than half of the world's population is at the risk of infection, and over 300 million people develop clinical disease each year of which 2 million are fatal [1]. Clinical malaria results from the replication of protozoan parasites of the genus *Plasmodium* in the circulating erythrocytes of the host. During the time between release from a rupturing mature schizont-infected erythrocyte and invasion of new erythrocytes, merozoites are transiently exposed in the circulation, and are thus potentially vulnerable to attack by preventive measures based upon immunological or biochemical methods. To design such tools, it is important to understand the molecular composition of the merozoite and the structure-function makeup of the molecular interactions that occur as the merozoite recognizes and gains entry into a host cell.

Like most apicomplexan parasites, the malaria merozoite invades host cells via a multistep process initiated by reversible binding to the erythrocyte surface. Subsequently, a high affinity attachment occurs between the apical end of the merozoite and the host cell, followed by the movement of the junctional adhesion zone (moving junction) around the merozoite toward its posterior pole. Finally the merozoite invaginates into the erythrocyte by forming a nascent parasitophorous vacuole [2]. The moving junction is one of the most distinctive features of apicomplexan invasion and was first observed in

*Abbreviations:* aa, amino acid(s); Ab, antibody; AMA1, apical membrane antigen 1; GST, Glutathione S transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RON, rhoptry neck protein.

☆ Sequence data from this article have been deposited with the GenBank™/EMBL/DDBJ databases under accession numbers AB444588–AB444592.

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*Plasmodium* species in the late 1970s [3], but the molecular nature of its structure remains unresolved.

Recent studies in *Toxoplasma gondii* suggest that host cell invasion involves protein discharge from at least two apical secretory organelles, the micronemes and rhoptries, based on the observation that a microneme protein, Apical Membrane Protein 1 (AMA1), forms a complex with three rhoptry neck (RON) proteins: RON2, RON4 and Ts4705 (RON5) [4–6]. These proteins have predicted orthologs in *P. falciparum*, and the RON4 ortholog has been reported to associate with *Pf*AMA1 [7] and to be localized at the moving junction [8], suggesting that the complex (and likely its function) is conserved between *T. gondii* and *P. falciparum* [7]. Attempts to knock-out the AMA1 gene locus were unsuccessful in both *Plasmodium* [9] and *T. gondii* [10], and the conditional reduction of *Tg*AMA1 expression severely impaired the cell invasion ability of *T. gondii* [11], indicating AMA1 has an essential function. The conservation of the RON proteins among apicomplexan parasites suggest that their functions and protein interactions are also conserved in the biology of host cell invasion. However, in *Plasmodium*, the details of this complex have yet to be fully characterized. In this study, to better understand the moving junction complex formation in *Plasmodium*, we sought to characterize *Pf*RON2 and determine the nature of its interaction with *Pf*RON4 and *Pf*AMA1.

## 2. Materials and methods

### 2.1. Malaria parasites

*P. falciparum* cloned lines 3D7, HB3, Dd2, 7G8, FVO, and D10 were maintained *in vitro*, essentially as previously described [12].

### 2.2. DNA and RNA isolation

Genomic DNA (gDNA) was isolated from *P. falciparum* using IsoQuick™ (Orca Research Inc., Bothell, WA). To determine transcription levels throughout the asexual stages, schizonts were purified by differential centrifugation on a 70%/40% Percoll-sorbitol gradient, after which released merozoites were allowed to invade uninfected erythrocytes for 4 h before the clearance of all remaining schizonts using 5% D-sorbitol. Fractions of the culture were harvested immediately and 24 h later, and then at 6 h intervals thereafter. Total RNA was isolated from parasite-infected erythrocytes stored at −20 °C in RNA*later*™ (Qiagen, Valencia, CA), using the RNeasy Mini Kit (Qiagen). Following DNase treatment, complementary DNA (cDNA) was generated with random hexamers using an Omniscript Reverse Transcription Kit (Qiagen).

### 2.3. Polymerase chain reaction (PCR) amplification and sequencing

A TBLASTN search was performed against the *P. falciparum* genome database (3D7 parasite line) via PlasmoDB website (http://www.plasmodb.org/) [13] using the *Tg*RON2 amino acid sequence as a query. To evaluate the polymorphism of *Pf*RON2, five pairs of overlapping primers were used for PCR amplification from HB3, FVO, Dd2, D10, and 7G8 parasite lines, and sequences were determined by direct sequencing of the PCR-amplified DNA fragments using an ABI PRISM® 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA). Oligonucleotides used were as follows: fRON2.F2 (5′-GATTCCAATAATTATAATTCTGATAATG-3′) and fRON2.R2 (5′-CGTAAAATATTCATTATATGAAAGATATGC-3′), fRON2.F3 (5′-GCATTAGGAGAACTTGTTGAACCA-3′) and fRON2.R3 (5′-CATAATATCTAAATAGGTTTTTGCTGAC-3′), fRON2.F4 (5′-GGATTAGTATTTTTATATGCAATGATTG-3′) and fRON2.R4 (5′-GTTATTTTCTAATAAATGTTTACTATCTTC-3′), fRON2.F5 (5′-GATAAATGGGATCAATTTATAAATAAGG-3′) and fRON2.R5 (5′-GCTAGCTACTGGTCCTGCACCT-3′), and fRON2.F6 (5′-ATGCAATTACCTTACTTAAGTCAAATG-3′) and fRON2.R6 (5′-ATATAAAATGAAAATAACAGAAAAGGTTATG-3′).

### 2.4. Quantification of pfron2 transcripts

Transcription of *ron2* was evaluated in the HB3 parasite line by real-time reverse transcription (RT)-PCR using a QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler System (Roche, Basel, Switzerland). As a control, transcription of *ama1* and *rhoph2* was also evaluated. Oligonucleotides used were as follows: fRON2.qF (5′-CAGAACTAAGCAAACATGTAAAACATG-3′) and fRON2.qR (5′-GTATAACGCCTTGCTCATTTCCTG-3′) for *pfron2* (product size is 133 bp); fAMA1.qF (5′-GGAAGAGGACAGAATTATTGGGAAC-3′) and fAMA1.qR (5′-CCTGAATCTTCTTGTTGGTATGTATG-3′) for *pfama1* (product size is 137 bp); fRhopH2.qF (5′-GTAACAACACTTACTAAGGCAGACT-3′) and fRhopH2.qR (5′-GTACAAAGCTACAATATTGTTAGATCT-3′) for *pfrhoph2* (product size is 210 bp). The same oligonucleotides were used to PCR-amplify DNA fragments to be ligated into the pGEM-T Easy® plasmid (Promega, Madison, WI) which was used to make a standard curve to evaluate the copy number of each transcript.

### 2.5. Antibodies

A DNA fragment encoding amino acid positions (aa) 21–98 of *Pf*RON2 was PCR-amplified from *P. falciparum* 3D7 gDNA and ligated into pEU-E01GST-N2, an expression plasmid with N-terminal glutathione S transferase (GST)-tag followed by a PreScission Protease cleavage site, designed specifically for the wheat germ cell-free protein expression system (CellFree Sciences Co., Ltd., Matsuyama, Japan) [14], to produce recombinant GST-fused fRON2N protein (GST-fRON2N). Oligonucleotides used in the PCR amplification were fRON2.SalF1 (5′-*GTCGAC*TCAGAACTAAGCAAACATGTAAAACATG-3′) and fRON2.SalR1 (5′-*GTCGAC*CCCATTATTCATTTCACTACCAGGA-3′) (SalI restriction sites are underlined). Produced GST-fRON2N was captured using a glutathione-Sepharose 4B column and eluted with 10 mM reduced gluthathione, pH 8.0. To generate anti-*Pf*RON2 sera, BALB/c mice were immunized subcutaneously with 20 µg of purified GST-fRON2N emulsified with Freund's adjuvant. A Japanese white rabbit was immunized subcutaneously with 500 µg of purified GST-fRON2N with Freund's adjuvant for the first time, followed by 250 µg thereafter. All immunizations were done 4 times at 3 week intervals, prior to collection of antisera. Rabbit anti-*Pf*RhopH2 serum was obtained from I. Ling (National Institute for Medical Research, UK) [15], Rabbit anti-*Pf*AMA1 serum was obtained from C. Long (National Institute of Health, USA), and mouse monoclonal anti-*Pf*RON4 antibody (Ab; 26C64F12) was obtained from J.-F. Dubremetz (Université de Montpellier 2, France) [7]. Rabbit anti-Clag3.1 serum was as previously described [16].

### 2.6. SDS-PAGE and Western blot analysis

The recombinant protein, GST-fRON2N, was digested with a PreScission Protease at 4 °C overnight before analysis. Triton X-100 extracts of *P. falciparum* or recombinant proteins were dissolved in SDS-PAGE loading buffer, incubated at 100 °C for 3 min, and subjected to electrophoresis under reducing conditions on a 5–20% polyacrylamide gel (ATTO, Japan). Proteins were then transferred to a 0.22 µm PVDF membrane (BioRad, Hercules, CA). The proteins were immunostained with antisera followed by horseradish peroxidase-conjugated secondary Ab (Biosource Int., Camarillo, CA) and visualized with Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on RX-U film (Fuji, Japan). The relative molecular sizes of the parasite-encoded proteins were calculated by reference to molecular size standards (BioRad).

### 2.7. Immunoprecipitation

Immunoprecipitation was carried out as previously described [17]. Briefly, proteins were extracted from late schizont parasite pellets by

1% Triton X-100 treatment in phosphate-buffered saline (PBS) containing cOmplete Proteinase Inhibitor Cocktail Tablets (Roche). Supernatants (50 µl) were pre-incubated at 4 °C for 1 h with 20 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). Recovered supernatants were incubated with rabbit antisera (anti-*Pf*RON2, anti-*Pf*AMA1, or anti-*Pf*RhopH2) or mouse anti-*Pf*RON4 Ab with gentle rotation at 4 °C for 2 h and then 20 µl of 50% protein G-conjugated beads were added. After 1 h 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 extracted from the protein G-conjugated beads by incubation with SDS-PAGE reducing loading buffer at 100 °C for 3 min. Supernatants were collected for Western blot analysis.

### 2.8. Indirect immunofluorescence assay

Thin smears of schizont-enriched *P. falciparum*-infected erythrocytes (Dd2 parasite line) were prepared on glass slides and stored at −80 °C. The smears were thawed, formaldehyde-fixed, and preincubated with PBS containing 5% non-fat milk at 37 °C for 30 min. They were then incubated with antisera at 37 °C for 1 h, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-(IgG and IgM) secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa546-conjugated goat anti-(IgG and IgM) secondary Ab (Invitrogen, Carlsbad, CA) at 37 °C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted in ProLong Gold antifade reagent (Invitrogen) and viewed under oil-immersion. High resolution image-capture and processing were performed using a confocal scanning laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). Images were processed in Adobe Photoshop (Adobe Systems Inc., San José, CA).

### 2.9. Immunoelectron microscopy

Parasites were fixed for 15 min on ice in a mixture of 1% paraformaldehyde–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 described [18,19]. Thin sections were blocked at 37 °C for 30 min in PBS containing 5% non-fat milk and 0.01% Tween 20 (PBS-MT). Grids were then incubated at 4 °C overnight with mouse anti-*Pf*RON2 or control sera in PBS-MT. 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-mouse IgG conjugated to 10 nm gold particles (Amersham Life Science, Arlington, IL) diluted 1:20 in PBS-MT, rinsed with PBS-BT, and fixed on ice for 10 min in 2.5% glutaraldehyde to stabilize the gold. Then the grids were rinsed with distilled water, dried, and stained with uranyl acetate and lead citrate. Samples were examined with a transmission electron microscope (JEM-1230; JEOL Ltd., Tokyo, Japan).

### 2.10. Primary structure analysis of the protein

Signal peptide sequence was evaluated by SignalP3.0 [20]. Transmembrane region was evaluated by TMpred [21] and TMHMM2.0 [22]. Low complexity region was evaluated by Globplot 2.3 [23]. Amino acid sequence alignment was generated by MUSCLE [24].

### 2.11. Statistical analysis

Number of nonsynonymous substitutions over numbers of nonsynonymous sites ($d_N$), number of synonymous substitutions over numbers of synonymous sites ($d_S$), and their standard errors were computed using the Nei-Gojobori method with Jukes-Cantor correction implemented in MEGA 4.0.1 [25]. Standard errors were estimated using the bootstrap method with 500 replications. The statistical difference between $d_N$ and $d_S$ was tested using a one-tail *Z*-test with 500 bootstrap pseudosamples.

## 3. Results

### 3.1. RON2 orthologs of apicomplexan parasites

Using *Tg*RON2 as a query in BLAST analyses [26], and similar analyses using the predicted orthologs thus identified, we found RON2 orthologs in *P. falciparum* (*Pf*RON2; PF14_0495, PlasmoDB), *P. yoelii* 17XNL strain (*Py*RON2; PY06813, TIGR), *P. knowlesi* H strain (*Pk*RON2; PKH_125430 or PK14_2335w, Sanger Centre), and *P. vivax* Sal-I strain (*Pv*RON2; Pv117880, TIGR), *P. berghei* (*Pb*RON2; Contig5108), *P. chabaudi* (*Pch*RON2; Contig882.0), *Theileria annulata* (*Ta*RON2; Fig. S1A, TA19445 and TA19390, Sanger Centre [27]), *Theileria parva* (*Tp*RON2; Fig. S1B, TP01_0014, TIGR [28]), and *Babesia bigemina* (*Bbig*RON2; Fig. S1C, Contig3449, Sanger Centre). The RON2 were fragmented in the *P. berghei*, *P. chabaudi*, *T. annulata*, and *T. parva* genome nucleotide sequence databases, and full-length versions were constructed (supplementary Table S1).

### 3.2. PfRON2 protein structure and similarity to RhopH1/Clag proteins

The full-length *Pf*RON2 protein consists of 2189 residues with a putative signal peptide sequence at its N-terminus from amino acid positions (aa) 1 to 20. An interspecies variable region (aa 55–878), exhibiting low complexity and many repeats [23], was identified by comparing 6 *Plasmodium* RON2 amino acid sequences (Figs. 1 and S2). A BLASTP search using the conserved region of *Pf*RON2 (aa 879–2189) as a query identified *P. vivax* RhopH1/Clag homolog (XP_001616939.1, aa 251–394; E=0.001) as possessing homology with *Pf*RON2 aa 1105–1259. A Position-Specific Iterated BLAST search using *Pf*RON2 aa 1105–1259 as a query converged at iteration 3 and indentified most of the RhopH1/Clag genes in *Plasmodium* species. Alignment of RhopH1/Clag with RON2 from multiple genera identifies a predicted globular domain that is likely stabilized by disulfide bonds between 4 conserved Cys residues (Fig. 2). Three transmembrane regions were predicted by TMpred, however TMHMM2.0 predicted only a single transmembrane region for all *Plasmodium* RON2 orthologs assessed.. Interestingly, TMpred predicted a putative transmembrane region in the region conserved between RhopH1/Clag and RON2 (Fig. 2). Because RhopH1/Clag is a component of a soluble protein complex, we considered that these predicted transmembrane regions in RhopH1/Clag and RON2 constitute a likely hydrophobic region buried within a globular domain. Another predicted transmembrane region at aa 1114–1133 in *Pf*RON2 is also possibly hydrophobic region buried within a globular domain. TMpred considers the observation that there is an overrepresentation of positively charged amino acid

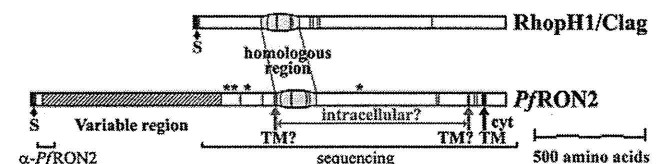


Fig. 1. Schematic representation of *Pf*RON2. S and TM indicate putative signal peptide (aa 1–20) and transmembrane sequences, respectively. The shaded box indicates an interspecies variable region. Vertical red bars indicate conserved Cys residues among orthologous sequences. Homologous region between RhopH1/Clag and RON2 is indicated by a yellow box. The region used to generate anti-*Pf*RON2 sera (α-*Pf*RON2) and the region sequenced in the laboratory lines (sequencing) are indicated. Asterisks indicate polymorphic sites.