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

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ABSTRACT

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.

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*CCCCATTATTCATTTCACTACCAGGA-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