

Review: Identification of *Plasmodium yoelii rhoph2* gene

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Introduction

Plasmodium spp. are obligate intracellular parasites throughout much of their life cycle and entry into host erythrocytes is a prerequisite for asexual blood stage development. *Plasmodium* merozoites invade erythrocytes, discharging the contents of their apical organelles, the micronemes, rhoptries and dense granules. Proteins located in these organelles, particularly erythrocyte binding proteins, are considered to be important for erythrocyte invasion and have been studied as vaccine targets, with the aim of inducing antibodies blocking invasion. For example, passive immunization with monoclonal antibodies (mAb) specific for a 235-kDa rhoptry protein [1], or active immunization with this protein [2], protect mice against blood stage challenge with *P. yoelii*. A number of rhoptry proteins have been identified, including a complex of high molecular mass proteins containing three distinct polypeptides, RhopH1, RhopH2 and RhopH3 (the RhopH complex) [3 – 6]. Antibodies against the *P. falciparum* RhopH (PfRhopH) complex partially inhibit growth of *P. falciparum* *in vitro* and *in vivo*, consistent with their potential as vaccine targets [7 – 9]. The genes for RhopH1, 2 and 3 have been cloned from *P. falciparum* and *P. yoelii* [10 – 13]. The PfRhopH3 gene appears to be essential for asexual development, as judged from unsuccessful attempts to disrupt the gene locus [14]. The genes encoding RhopH1 in *P. falciparum* and *P. yoelii* are members of a gene family that was originally defined by a cytoadherence-linked asexual gene on chromosome 9 (*clag9*) [15, 16]. *clag9* was initially proposed to encode a protein involved in the binding of infected erythrocytes to host endothelial cells, whereas our recent studies indicate that *clag9* gene product is involved in the RhopH complex, suggesting a function in the remodeling of the infected erythrocyte following invasion by the merozoite [17].

Part of the work presented in the Forum Cheju-9 (Oct, 2003) is currently under consideration for the publication, thus, in this proceeding we will present the identification of *P. yoelii* RhopH2 gene, which was only briefly described in the reference 13.

Materials and methods

Monoclonal antibody production

P. yoelii 17X (lethal) was maintained in Crj:CD-1 mice (Charles River Japan Inc., Japan). Mature *P. yoelii* schizonts were enriched by differential centrifugation on 45% Percoll (Amersham Pharmacia Biotech Inc., U. K.) and disrupted by nitrogen decompression to release organelles. The organelles were purified by sucrose density gradient centrifugation and the rhoptry-rich fraction was selected after identification by negative staining electron microscopy. This fraction, containing 100 µg of protein, was injected intraperitoneally into an 8-week old female BALB/c mouse, together with Freund's complete adjuvant on the first occasion and with Freund's incomplete adjuvant 3 weeks later. Six weeks after the initial injection, an intravenous boost with the same amount of rhoptry-rich fraction in phosphate buffered saline (PBS, pH 7.4) was administered, and the spleen was used to produce hybridoma cell lines. After an initial screening by immunofluorescence microscopy using acetone-fixed smears of mature *P. yoelii* schizonts, hybridoma cell lines were cloned by two rounds of limiting dilution. Cloned cell lines were expanded as ascites in mice primed with pristane (Wako, Japan), and immunoglobulins were purified from ascitic fluid using an Ampure PA kit (Amersham Pharmacia Biotech Inc.).

Affinity purification of ~140-kDa P. yoelii proteins and peptide sequencing

Preparations of enriched mature *P. yoelii* schizonts were lysed in extraction buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM EDTA, 0.2% NP40, pH 7.4, containing 1 µg ml⁻¹ leupeptin, 1 µg ml⁻¹ pepstatin A, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Wako)) for 1 h. The lysate was centrifuged at 15,000 × g for 10 min at 4°C, and then *P. yoelii* RhopH (PyRhopH) complex proteins were purified by affinity chromatography using MAb #25 bound to Formyl-Cellulofine (Seikagaku-Kogyo, Japan) from the parasite-lysate. The purified protein complex was resolved by 7.5% SDS-PAGE under reducing conditions, and the bands around 135 – 140 kDa were excised. The extracted proteins were then digested overnight with lysyl-endo-peptidase at 37°C in 500 mM Tris-HCl buffer. Peptide fragments were fractionated by reverse phase high-performance liquid chromatography and analyzed on a protein sequencer (PSQ-1 system; Shimadzu, Japan).

DNA and RNA isolation and construction of the cDNA library for P. yoelii

Genomic DNA (gDNA) was isolated from *P. yoelii* using IsoQuick™ (Orca Research Inc., Bothell, WA). Total RNA was isolated from schizont-enriched *P. yoelii*-infected erythrocytes using Isogen (Nippon Gene, Japan) and dissolved in DEPC-treated water. Complementary DNA (cDNA) was generated with random hexamers using a GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA) and the cDNA library was constructed with lambda Zap® II vector according to the manufacturer's instruction (Stratagene, La Jolla, CA).

PCR and sequencing strategy

The *P. yoelii* gene coding for RhopH2 was identified by TBLASTN analysis of preliminary sequence data in the *P. yoelii* genome database obtained from The Institute for Genomic Research website (<http://www.tigr.org>) [18]. The amino acid sequences of peptides identified in lysyl-endopeptidase digestion products of affinity-purified protein were used in this search. The cDNA sequence was determined by direct sequencing (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems) of the RT-PCR products of *P. yoelii* mRNA and the locations of introns were identified by comparison of cDNA and gDNA sequences in the database.

Polyclonal antibody production to PyRhopH2

Based on the deduced amino acid sequences of PyRhopH2, two synthetic peptide dendrimers (peptide B, QNKKEYQRKQREEII (amino acid residues (aa) 986–1000) and peptide C, THKPQFNFPENSNYR (aa 1296–1310)) were made using a tetravalent multiple antigenic peptide (MAP) system (ABI 432A PeptideSynthesizer; Applied Biosystems, Foster City, CA). A mouse (BALB/c, 8-week-old female; Charles River Japan Inc.) was immunised intraperitoneally with 50 µg of peptide B in Freund's complete adjuvant. After 3 weeks the mouse was boosted with the same amount of peptide in Freund's incomplete adjuvant on four occasions at 2-week intervals, generating antiserum α -yRhH2B. A rabbit (Japanese White) was also immunised using 200 µg of peptide C in adjuvant, administered by subcutaneous injection, to produce antiserum α -yRhH2C.

SDS-PAGE, Western blot analysis, and Indirect IFA microscopy

For Western blotting of *P. yoelii* proteins, affinity-purified proteins were dissolved in SDS-PAGE loading buffer, incubated at 85°C for 10 min, and subjected for the electrophoresis on a 7.5% polyacrylamide gel (ATTO, Japan). Proteins were then transferred from the gel to a 0.22 µm PVDF membrane (Bio-Rad, Hercules, CA). The proteins were either visualized with SYPRO Ruby protein gel stain (Molecular Probes Inc., Eugene, OR), or immunostained with mouse or rabbit serum followed by horseradish peroxidase-conjugated goat second antibody (Biosource Int, Camarillo, CA) and visualized with ECL plus (Amersham Pharmacia Biotech Inc.) on RX-U film (Fuji, Japan).

For Immunofluorescence microscopy, thin smears of schizont-rich *P. yoelii*-infected mouse erythrocytes were prepared on glass slides and stored at –80°C. Slides were thawed, acetone-fixed, preincubated with PBS containing 5% non-fat milk at 37°C for 30 min, reacted with rabbit antiserum at 37°C for 1 h, followed by FITC-conjugated goat anti-rabbit (IgG and IgM) antibody (Biosource Int.) at 37°C for 30 min. The differential interference contrast and FITC-stained images were collected using a fluorescence microscope (BX50; Olympus, Japan) with charged coupled device camera (DC500; Leica Microsystems, Germany), and processed using Adobe PhotoShop.

Results

Peptide sequences of 135- and 140-kDa protein in the RhopH complex from P. yoelii

The RhopH complex was affinity-purified from extracts of *P. yoelii*-infected erythrocytes using mAb #25, which recognises a 140-kDa protein, as described previously [12], and used for peptide sequence analysis. Among 10 peptide sequences obtained, four sequences were identified as a part of PyRhopH1A and reported previously [12]. The gene coding for PyRhopH2 was identified using the remaining six peptide sequences as queries in a TBLASTN search of the *P. yoelii* genome database and a DNA sequence coding for a protein (PyRhopH2) was identified (Table 1 and Fig. 1A). Oligonucleotides were designed using gDNA sequence and used to obtain cDNA sequence for *pyrhoph2* (the sequence data is available in the GenBank™/EMBL/DDBJ under accession number AB075605). Figure 1A shows the positions of the exon/intron boundaries for *P. yoelii* and *P. falciparum rhoph2*, which was independently identified by Ling and Holder (U. K.). The overall structure of the gene in *P. yoelii* and *P. falciparum* is very similar, each gene containing nine introns in similar locations. PyRhopH2 are predicted to have a signal sequence at the N-terminal end and encodes multiple Cys residues at the conserved location between PfRhopH2, but otherwise no structural or sequence similarity to known proteins in the public databases was identified.

pyrhoph2 encodes a 140-kDa protein located at the merozoite apical end

The identity of the *pyrhoph2* was confirmed by Western blot analysis of sera raised against synthetic peptides based on the deduced amino acid sequence of PyRhopH2. As is the case with mAb #25, a 140-kDa protein was recognised (Fig. 1B). The rabbit serum raised against peptide C from PyRhopH2 gave the punctate pattern of fluorescence with *P. yoelii* schizonts, typical of antibodies reacting with apical organelles, similar to that obtained with mAb #25 (Fig. 1C).

Table 1. Peptide amino acid sequences obtained from *Plasmodium yoelii* (17XL) affinity-purified rophtry proteins

Fraction name	Amino acid sequence determined by peptide sequencing ^a	Corresponding translated sequence from <i>P. yoelii</i> 17XL ^b
R77	DFHIYGLYFDLE ^c	¹⁸⁸ DFHIYGLYFDLE
R56	LIHEVVPEDGNIEE ^c	³⁰⁶ LIHEVVPEDGNIEE
R65	SNXXRHVLVSRNXILK	⁵⁵⁷ SNFIRHVLVSRNFILK
R68	ELXENVXXI	¹⁰⁰⁴ ELDENVTEI
R60	YSXDFLERTVY	¹¹⁴⁵ YSRDFLERRVY
R66#1	VFIHSSFIHNLTK ^c	¹²⁴³ VFIHSSFIHNLTK

^a *P. yoelii* RhopH2 was affinity-purified, digested with lysyl-endopeptidase and the peptide fragments were fractionated by reverse-phase high-performance liquid chromatography and analysed on a protein sequencer. X, undetermined amino acid.

^b The number at the start of a peptide gives the position of the leading amino acid in the translated sequences of *P. yoelii* RhopH2.

^c These sequences were used in TBLASTN search of the *P. yoelii* database and gave a strong match with a single sequence in a contig (TIGR c5m1675)

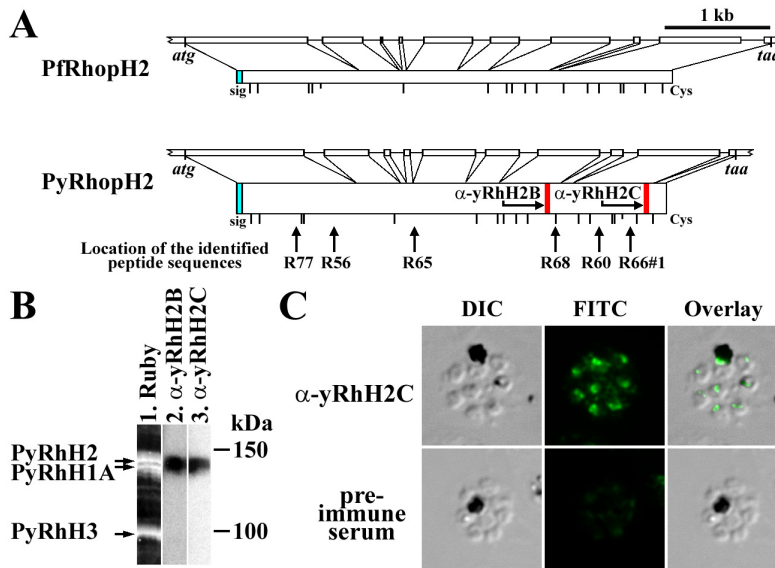


Fig 1. (A) The positions of the exon/intron boundaries for *P. yoelii* and *P. falciparum rhopH2*. Start (atg) and stop (taa) codons, predicted signal sequence (sig) are indicated. Cys residues are indicated with vertical bars under the scheme, longer bar indicate conserved Cys residues between two orthologs. Regions used to generate antisera and locations of the identified peptide sequences are also indicated. (B) Affinity-purified proteins were subjected for the electrophoresis on a 7.5% polyacrylamide gel and visualized with SYPRO Ruby protein gel stain (lane 1), or transferred to a PVDF membrane and immunostained with mouse anti-yRhH2B (lane 2) or rabbit anti-yRhH2C (lane 3) serum and visualized with ECL plus. A 140-kDa protein was recognised by these sera. (C) Immunofluorescence microscopy. Thin smears of schizont-rich *P. yoelii*-infected mouse erythrocytes were reacted with rabbit anti-yRhH2C serum and visualized with FITC. The punctate pattern of fluorescence, typical of antibodies reacting with apical organelles was observed. Control rabbit preimmune serum detect no signal. DIC, differential interference contrast.

Discussion

By compared with the increasing knowledge of the microneme function in the parasite gliding motility (reviewed by [19]), little is known about the rhoptry function. It appears that *P. falciparum* RhopH3 is transferred on invasion to the parasitophorous vacuole membrane (reviewed by [20]) and to persist throughout the development of ring stage parasites. Recently RhopH2 was detected on the erythrocyte cytosol side of the parasitophorous vacuole membrane in *P. falciparum* [21]. The complex is not disrupted during transfer suggesting that its integrity is important for function. Furthermore the transfer of the PfRhopH complex during invasion and its location in newly invaded erythrocytes would suggest that its role is post-invasion.

We have now identified the gene coding for the last member of the RhopH complex. Therefore, we have the basic tools for studying each protein within the complex, both singly and in combination with each other. Recently we identified PyRhopH complex specifically bound GPI-anchored molecules on the mouse erythrocyte surface using newly developed semi-quantitative erythrocyte binding assay based on the flow cytometry [Rungruang et al, under submission]. It will be important to determine the regions within the separate molecules that bind with each other and host erythrocyte surface molecules.

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