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Characterization of the high molecular mass rhoptry protein, RhopH, complex, in *Plasmodium yoelii*

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INTRODUCTION

Plasmodium spp. are obligate intracellular parasites and entry into host erythrocytes is a prerequisite for asexual-stage development. *Plasmodium* merozoites invade erythrocytes, discharging the contents of their apical organelles, the micronemes, rhoptries, and dense granules. The molecules 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 to block invasion. For example, passive immunization with monoclonal antibodies (MAb) specific for a 235-kDa rhoptry protein (Freeman *et al.*, 1980), or active immunization with the protein (Holder and Freeman, 1981), 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 (the RhopH complex) containing three distinct polypeptides, RhopH1, RhopH2 and RhopH3 (Campbell *et al.*, 1984; Holder *et al.*, 1985; Lustigman *et al.*, 1988; Hienne *et al.*, 1998). 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 (Siddiqui *et al.*, 1987; Cooper *et al.*, 1988; Doury *et al.*, 1994). The PfRhopH complex is considered to have a critical role in erythrocyte invasion because the complex binds the erythrocyte and distributes into the erythrocyte and parasitophorous vacuolar membranes (Sam-Yellowe *et al.*, 1988; Sam-Yellowe and Perskins, 1991). The gene for RhopH3 has been cloned from *P. falciparum* (Brown and Coppel, 1991), and the protein appears to be essential because attempts to disrupt the PfRhopH3 gene locus were unsuccessful (Cowman *et al.*, 2000). The genes encoding RhopH1 and RhopH2 have not been

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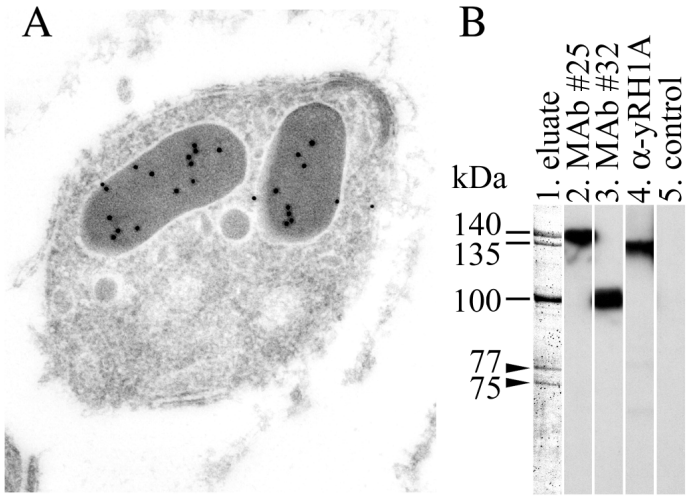

Fig. 1. (A) Immunoelectron microscopy of a merozoite-stage *Plasmodium yoelii* parasite with MAb #25. The gold-particles were seen on the body of the rhoptries, and no gold particles were seen on the micronemes or dense granules. (B) Western immunoblot analysis of the affinity purified *P. yoelii* rhoptry proteins under reducing conditions. The proteins were visualized with Coomassie Brilliant Blue G-250 (lane 1) or immunostained with MAbs #25, #32, anti-PyRhopH1A serum, or control serum for PyRhopH1A (lanes 2-5, respectively). These antibodies and antiserum also reacted under non-reducing conditions. The sizes of 3 bands characteristic for the *Plasmodium* RhopH complex (bar) and 2 uncharacterized bands (arrowhead) are indicated.

identified yet in any *Plasmodium* species.

In this review article, we describe the generation of monoclonal antibodies against *P. yoelii* rhoptry proteins, and identification of the genes encoding RhopH1 and RhopH3.

Monoclonal antibodies

MAbs against *P. yoelii* rhoptry proteins were generated as described elsewhere (Kaneko *et al.*, 2001). Cloned hybridoma 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., UK). By immunofluorescence microscopy, MAbs #25 [IgG1 (λ)] and #32 [IgG2b (κ)] showed a punctate pattern in mature schizonts, typical for an antibody reacting with the apical organelles of *Plasmodium* merozoites. MAb #25 reacted with

the body of rhoptries by immunoelectron microscopy (Fig. 1A)

Western immunoblot analysis against *P. yoelii* proteins extracted from mature schizonts revealed that the 140-kDa protein reacted with MAb #25, whereas the 100-kDa protein reacted with MAb #32. Because the molecular masses of these proteins are similar to those of the proteins forming the RhopH complex in *P. falciparum* (Holder *et al.*, 1985), we checked whether or not these proteins form a complex. Affinity purification using MAb #25 co-purified 5 bands at 140, 135, 100, 77, and 75 kDa. The 3 bands around 140, 135, and 100 kDa are a characteristic feature of the RhopH complex in *P. falciparum* (Fig. 1B, lane 1). Immunostaining of the affinity-purified proteins using MAb #25 revealed that the 100-kDa protein reacted with MAb #32 (Fig. 1B, lanes 2 and 3).

Identification of genes encoding RhopH complex in *P. yoelii*

To obtain the sequence of the genes encoding the *P. yoelii* 140-, 135- and 100-kDa rhoptry proteins, partial peptide sequences were determined. The protein complex was purified from mature schizonts by affinity chromatography using MAb #25 followed by SDS-PAGE. The bands at 140, 135, and 100 kDa were excised from the gel, digested with lysyl-endopeptidase, and peptide fragments were fractionated by reverse phase high-performance liquid chromatography and analyzed on a protein sequencer (PSQ-1 system; Shimazu, Japan). Sequencing of peptides from the purified 135- and 140-kDa proteins identified 10 distinct amino acid sequences and the purified 100-kDa proteins identified 5 distinct amino acid sequences (Table

Table 1. Amino acid sequences of peptides derived from PyRhopH1A and PyRhopH3, which were obtained from the purified *Plasmodium yoelii* 135-kDa, or 100-kDa rhoptry proteins

Fraction name	Peptide amino acid sequence	Corresponding sequence and location	
PyRhopH1A (135-kDa proteins)[a]			
R49	YQLSLISRTYGDEFQ	NLIELHDTFNISSK	(335-349)[b]
R52	DDNVLFEDVDKEEEF	YQLSLISRTYGDEFQ	(1271-1285)
R66#2	NEYNEIXNARXSDTNFK	DDNVLFEDVDKEEEF	(1224-1240)
R67	NLIELHDTFNIXXK	NEYNEIINARLSDTNFK	(757-770)
PyRhopH3 (100-kDa protein)			
R51	IDDFDFK	IDDFDFK	(407-413)
R52	YNEEWLPK	YNEEWLPK	(725-732)
R57	FLTTNELSFTK	FLTTNELSFTK	(296-306)
R61	SSNIFVDAFQK	SSNIFVDAFQK	(373-383)
R62	IFSIFK	IFSIFK	(384-389)

[a]Only amino acid sequences of peptides derived from PyRhopH1A are shown.
[b]Locations are indicated with amino acid number.

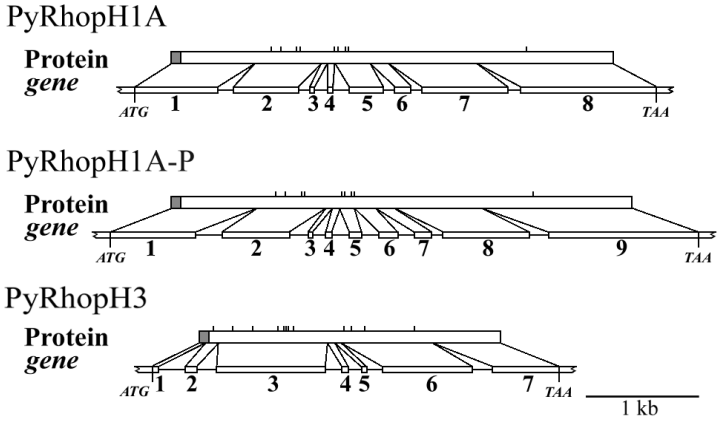

Fig. 2. Gene structure of PyRhopH1A, 1A-P, and 3. Cysteine residues are indicated with the vertical bar above the schematics.

1).

For 135-kDa and 100-kDa bands, DNA fragments were PCR-amplified from cDNA library of *P. yoelii* 17X (lethal) schizonts with combinations of degenerate oligonucleotides, sequenced, and the full sequence of the putative open reading frame of the cDNA was completed. Genomic DNA (gDNA) sequence was obtained from PCR products amplified from splinkerette genomic libraries of *P. yoelii* using gene-specific and splinkerette-specific primers. For 140-kDa band, gDNA sequence was obtained by BLASTN analysis using partial peptide sequences and gDNA and cDNA sequence was confirmed (manuscript in preparation).

For 135-kDa protein, we completed the cDNA and gDNA sequences encoding an open reading frame of 1292 amino acids in 8 exons (Fig. 2, PyRhopH1A). The deduced amino acid sequence has a putative secretory signal peptide at amino acid (aa) residues 1-24. The four peptide sequences (R49, R52, R66#2, and R67) were located in this protein sequence. Several of the other peptide sequences were identified by BLAST analysis of the *P. yoelii* genome database (TIGR) in another gene homologous to that encoding *P. falciparum* RhopH2 (Holder AA, personal communication).

For 100-kDa protein, comparison of the 4018 nucleotides of the gDNA to the corresponding cDNA revealed a 2649 nucleotide open reading frame in 7 exons, which is consistent with the structure of pfrhoph3. SignalP software predicted a putative signal peptide sequence at aa 1-24 (Fig. 2, PyRhopH3). Based on ClustalW analysis of the PyRhopH3 with PfRhopH3 amino acid similarity followed by manual adjustment for best fit, the RhopH3 protein was divided into 3 types of block: conserved blocks (70.0-86.4% similarity and 42.3-65.2% identity; aa 24-220, 231-262, 274-299, 398-457, 475-540, and 560-651 of PyRhopH3), semi-conserved blocks (45.6-57.5% similarity and 21.5-39.7% identity; aa 300-397, 652-730, and 749-821), and vari-

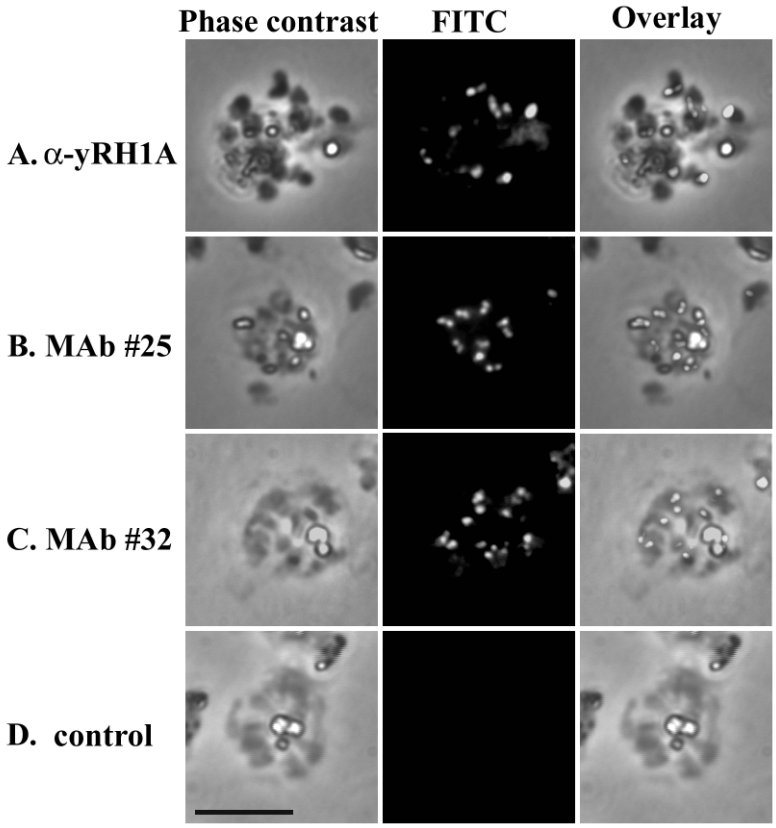

Fig. 3. Air-dried segmented schizont-stage parasites fixed with ice-cold acetone and reacted with anti-PyRhopH1A serum (row A), showing a typical punctate pattern for the apical organelle, similar to the images with MAbs #25 and #32 (rows B and C, respectively). Control serum gave no signal (row D). Phase contrast and FITC images were merged in the right panel (Overlay). The bar indicates 10 μm.

able blocks (5.3-27.8% similarity and 5.3-22.2% identity; aa 221-230, 263-273, 458-474, 541-559, 731-748, and 822-882). The conserved blocks are clustered at the N-terminal and central region, whereas the C-terminal region is variable. Twelve cysteine residues located in the conserved blocks are conserved between PyRhopH3 and PfRhopH3, suggesting a conformational role of these residues and a potential functional importance for these blocks (Shirano *et al.*, 2001).

Antisera generated based on the deduced amino acid sequences of PyRhopH1 and PyRhopH3 reacted to the 135- and 100-kDa band of the purified PyRhopH complex, respectively (Fig. 1B, lane 4 for PyRhopH1A). Immunofluorescence microscopy revealed that these antisera reacted at the apical end of the merozoites (Fig. 3). These findings confirmed that

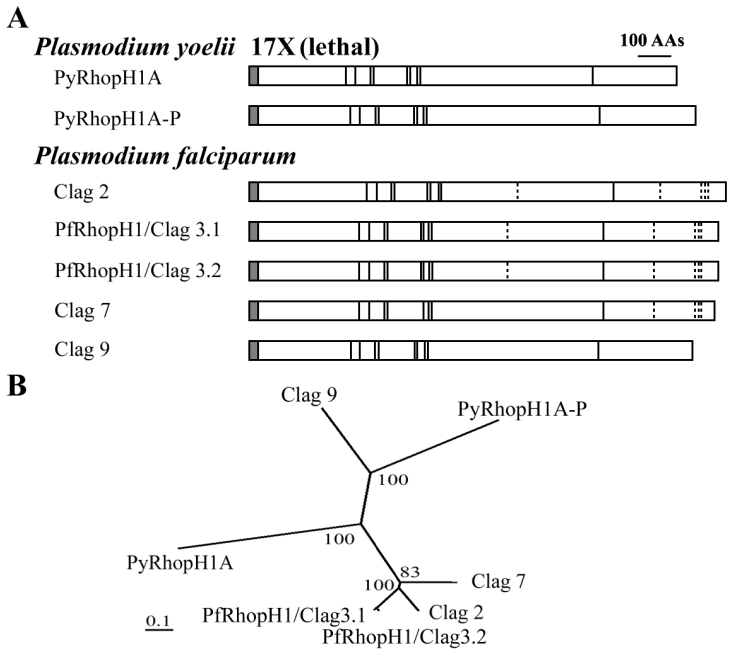

Fig. 4. *Plasmodium* RhopH1/Clag family. (A) The deduced amino acid sequence of Clag 2 was based on the exon/intron structure predicted by PfAGSS from *P. falciparum* chromosome 2 (Gardner *et al.*, 1998; Huestis *et al.*, 2001). The sequences for PfRhopH1/Clag 3.1, 3.2, and Clag 9 were obtained from CAB10571-2 and AF055476, respectively. The sequence for Clag 7 is after Holt *et al*. (2001). Conserved Cys residues in all members (solid vertical line) and among PfRhopH1/Clag 2, 3.1, 3.2, and/or 7 (broken lines) are indicated in the schematics. (B) Phylogenetic tree based on the amino acid sequences of the RhopH1/Clag multigene family. Bootstrap values per 100 are indicated.

cloned genes were encoding proteins of PyRhopH complex.

PyRhopH1 is a member of a multigene family (RhopH1/Clag family)

TBLASTN analysis of the *P. yoelii* genome database (TIGR) revealed the existence of a paralogue of the pyrhoph1 gene we had cloned (identified as c2m3539 in the TIGR database). Thus we designated the gene identified from the peptide sequences as pyrhoph1a and the second gene found in the TIGR database as pyrhoph1a-paralogue (pyrhoph1a-p). Using oligonucleotides designed on the gDNA sequence of pyrhoph1a-p, the cDNA sequence was determined (Accession number AB060735). The deduced amino acid sequence showed that PyRhopH1A-P was a 1350-amino acid residue protein with a signal peptide (aa 1-22) similar to PyRhopH1A.

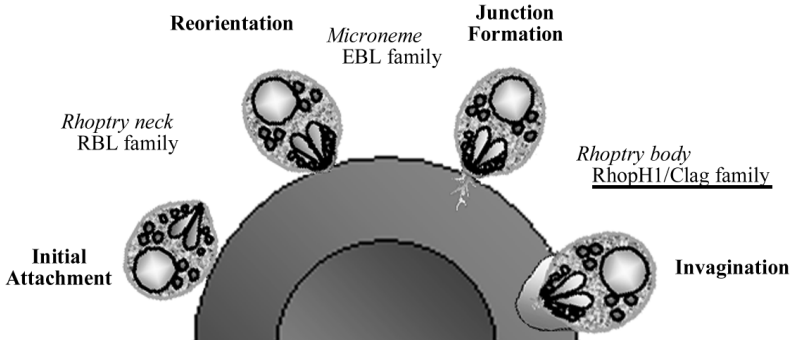

Fig. 5. Schematic of the early stages of erythrocyte invasion by the malaria merozoite, and the putative roles of the protein encoded in the multigene family.

A comparison of the cDNA sequence and the gDNA sequence in the database revealed that the open reading frame of pyrhoph1a-p was encoded in 9 exons. The 5th intron in pyrhoph1a-p is present at a location corresponding to the middle of the 5th exon of pyrhoph1a (Fig. 2, PyRhopH1A-P).

TBLASTN analysis of the *P. falciparum* genome databases revealed that PyRhopH1 was homologous to members of the *P. falciparum* cytoadherence linked asexual gene (clag) family, which was originally defined by a cytoadherence linked asexual gene on chromosome 9 (clag 9) (Holt *et al.*, 1999; Trenholme *et al.*, 2000), proposed to encode a protein involved in the binding of infected erythrocytes to host endothelial cells. However Holder's group in UK, independently identified a member of the clag family, clag3.1 or 3.2, encoding RhopH1 in *P. falciparum* by MALDI-ToF analysis. Thus, we propose to rename the genes encoding the *P. falciparum* 155-kDa rhoptry proteins (PfRhopH1) pfrhoph1/clag 3.1 and 3.2. Therefore the clag gene family is renamed as the rhoph1/clag gene family. To compare the sequences, all members of RhopH1/Clag family described in the literature were aligned. Ten Cys residues, including one at residue 24 in the putative signal sequence of PyRhopH1A, are conserved in all members, while 1 Cys residue in the central part are only found among Clag 2 and PfRhopH1/Clag 3.1 and 3.2 and 4 Cys residues at the C-terminus are only found among Clag 2, Clag 7, and PfRhopH1/Clag 3.1 and 3.2 (Fig. 4A). Among 5 rhoph1/clag members identified so far in *P. falciparum*, Clag 9 has phylogenetically distant from other members (Fig. 4B).

The multigene families localized in the microorganelles of the merozoites may play a critical role in the redundancy of the invasion of the host erythrocyte. At the neck of the rhoptry, a family called reticulocyte binding-like (rbl) has been identified, including 5 members in *P. falciparum* (pfrh1, pfrh2a, pfrh2b, pfrh3, and pfrh4) (Rayner *et al.*, 2000 & 2001; Taylor *et al.*, 2001; Triglia *et al.*, 2001; Kaneko *et al.*, 2002). In the microneme, another family called ery-

throcyte binding-like (ebl) has been identified, including 5 members in *P. falciparum* (jesebl, pebl, eba-175, baebl, and ebl-1) (Michon *et al.*, 2002). In addition to these families, we identified a new family (rhoph1/clag) localized at the body of the rhoptry (Fig. 5). Using multiple multigene families at a different step during the invasion, malaria parasites have a potential to invade into a variety of host erythrocytes. Further investigation is currently conducted to clear the role of the multigene family in the alternative invasion pathway of the malaria parasites.

ACKNOWLEDGEMENTS

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