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Sequence analysis of the Rhop-3 gene of *Plasmodium berghei* and *P. chabaudi*, reactivity of Rhop-3 protein within isolated rhoptries and binding of Rhop-3 to mouse erythrocytes

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ABSTRACT

The 110 kDa Rhop-3 rhoptry protein of *Plasmodium falciparum* is secreted into the erythrocyte membrane during invasion. It is an erythrocyte binding protein that is non-covalently associated with two other proteins, the 140 kDa Rhop-1 and the 130 kDa Rhop-2. We identified the Rhop-3 gene homologue in *P. yoelii* and demonstrated that the C-terminus is highly conserved. In order to identify the Rhop-3 gene homologue in *P. berghei* and *P. chabaudi*, a set of primers were designed based on the cDNA sequence of clone Y1412 of *P. yoelii* and used to amplify genomic DNA from *P. berghei* and *P. chabaudi* by polymerase chain reaction (PCR). Analysis of the DNA and deduced amino acid sequence demonstrated sequence homology to *P. falciparum* Rhop-3. We examined the distribution of Rhop-3 epitopes within isolated rhoptries of the three rodent *Plasmodium* species and *P. falciparum*, and investigated the erythrocyte binding property of rodent *Plasmodium* Rhop-3. We also evaluated the immunogenicity of isolated rhoptries from the rodent *Plasmodium* species following treatment of the organelles with 100 mM sodium carbonate, pH 11.5 and

0.5 % SDS, to stimulate antibodies to proteins not accessible when untreated organelles are used for immunization. We show that the integrity of the isolated organelles was stable and the organelles were reactive with Rhop-3 specific antisera. In addition, the rodent *Plasmodium* Rhop-3 protein bound to mouse erythrocytes and was recognized by Rhop-3 specific antibody. Furthermore, treated organelles stimulated antisera that recognized additional rhoptry proteins not seen when antisera prepared against whole untreated organelles were examined by western blotting. Taken together, these data suggest that malaria vaccine studies using the Rhop-3 protein can be performed directly in vivo using the rodent *Plasmodium* models.

INTRODUCTION

Investigations regarding the identification of vaccine molecules of rhoptry origin rely on the conservation of rhoptry protein structure and conservation in the properties of the proteins across *Plasmodium* species (reviewed in Perkins 1992; Sam-Yellowe 1996; Dubremetz et al. 1998; Kappe et al. 1996). In an effort to understand the role of the rhoptries during invasion, rhoptries have been isolated from different Apicomplexans in order to analyze the contents of the organelle, determine host immunological response to organelle contents, and to characterize the molecular structure of rhoptry genes (Dubremetz et al. 1989; Leriche and Dubremetz 1991; Etzion et al. 1991; Machado et al. 1993; Tomley et al. 1994; Sam-Yellowe et al. 1998). A number of rhoptry genes characterized exist as multigene families (Borre et al. 1995; Carcy et al. 1994; Dalrymple 1993).

In previous studies to determine the role of rhoptry proteins during invasion, we showed that the *P. falciparum* rhoptry protein Rhop-3, is secreted into the erythrocyte membrane during merozoite invasion (Sam-Yellowe et al. 1988). The Rhop-3 protein along with Rhop-1 and Rhop-2, the other members of the high molecular weight rhoptry protein complex of Rhop-H bind to erythrocyte membranes and inside-out-vesicles of human and mouse erythrocytes (Sam-Yellowe and Perkins 1990; 1991), suggesting their involvement in the invasion process. Rhop-3 specific antibodies inhibit invasion in vitro (Sam-Yellowe and Perkins 1990) and immunization with the high molecular weight protein complex, Rhop-H was partially protective to a lethal challenge infection with *P. falciparum* in *Aoutus* monkeys (Siddiqui et al.1987).

For proteins with vaccine potential, it is important to determine the extent of sequence variability. Rhop-3 sequence variability has not been investigated. The

conservation of rhoptry proteins is in marked contrast to the large diversity observed for other asexual stage antigens such as MSP-1, the S-antigen and PfEMP-1 (Cowman et al. 1995; Miller et al. 1993; Su et al. 1995). Based on the conservation of rhoptry proteins and their participation during merozoite invasion, rhoptry proteins are considered important vaccine candidates against malaria (Facer and Tanner 1997).

In order to investigate the immunological significance of the Rhop-3 protein in vivo, we have been interested in determining the extent of Rhop-3 gene conservation among rodent *Plasmodium* species. We reported on immunological cross reactivity of rhoptry specific antibodies with putative novel rhoptry proteins of *P. yoelii*, *P. berghei* and *P. chabaudi* (Sam-Yellowe et al. 1998), we showed that the morphology of the isolated organelles is the same among the three rodent *Plasmodium* species (Sam-Yellowe et al. 1999), and we identified the Rhop-3 gene homologue in *P. yoelii* (Anthony et al. 2000). However, it was not clear to what extent the Rhop-3 gene was conserved among the rodent *Plasmodium* species and if the rodent *Plasmodium* Rhop-3 protein shared the properties of erythrocyte binding with the *P. falciparum* protein.

The objectives of the current study were two fold; 1) to identify the Rhop-3 gene homologues in *P. berghei* and *P. chabaudi* and 2) to determine whether the rodent *Plasmodium* Rhop-3 is an erythrocyte binding protein and if the protein is similarly distributed within the isolated rhoptries. As part of our ongoing studies to identify additional rhoptry proteins we also investigated the immunogenicity of rhoptries treated with sodium carbonate and SDS. We demonstrated extensive amino acid similarity in the C-terminus of Rhop-3 protein among the rodent *Plasmodium* species. In addition, the rodent *Plasmodium* Rhop-3 protein binds mouse erythrocytes and isolated rhoptries were reactive with Rhop-3 specific antibodies. Rhop-3 involvement in parasite development and blood stage immunity can now be studied directly in vivo.

MATERIALS AND METHODS

Parasite maintenance:

Plasmodium yoelii (strain 17 XL), *P. berghei* (K-173), and *P. chabaudi adami* infected mice were maintained as described (Sam-Yellowe and Judd 1990; Sam-Yellowe et al. 1998), with the latter kept on a reversed day-night cycle, with

light provided from 1800 h-0600 h. Infected erythrocytes (1 x 10^6 cells/ml) were used for weekly maintenance of the parasites in CFW (Carworth Farm White) mice of mixed sex, bred and maintained at the Cleveland State University animal facilities. Synchronized parasites from 65 % Percoll gradients (Pasvol et al. 1978) were used to initiate the infections for fractionation experiments. *Plasmodium falciparum* (FCR-F86), a gift from Dr. Naomi Lang-Unnasch (University of Alabama, Birmingham, AL) was maintained according to the method of Trager and Jensen (1976). Parasites were grown in human type A^+ erythrocytes (American Red Cross, Cleveland Branch, OH) at 5 % hematocrit in RPMI 1640-Hepes medium supplemented with 10 % pooled human serum (American Red Cross) and 20 mM glucose.

Rhoptry isolation:

Rhoptry isolation from segmented schizont-infected erythrocytes was performed as described (Sam-Yellowe et al. 1998). Fractions for electron microscopy from *P. falciparum* and the three rodent *Plasmodium* species were pooled from a density range of 1.12-1.18 g/ml diluted in disruption buffer (DB) (10mM KCl, 10mM Hepes, 1 mM EDTA. 250 mM sucrose, pH 7.4) and centrifuged 60, 000 rpm for 1 h in an SW-60 rotor (Beckman). The rhoptry pellet was resuspended gently in phosphate buffered saline (PBS), centrifugation repeated for 30 min at 60,000 rpm, the supernatant discarded and the pellet fixed for immunoelectron microscopy in 1 % formaldehyde containing 0.1 % gluteraldehyde in 0.1 M phosphate buffer pH 7.4. The pellet was embedded in LR white resin (Polyscience Inc., Warrington, PA) and processed for immunoelectron microscopy using antiserum #686 as described (Yang et al. 1996). Fixed segmented schizont-infected erythrocytes were also processed for IEM using antiserum prepared against sodium carbonate or SDS treated rhoptries using previously described methods (Yang et al. 1996).

Mouse immunizations:

Gradient fractions of density 1.12-1.18 g/ml from each of the three rodent *Plasmodium* species were pooled separately, dialysed, lyophilized and divided in two groups. One group was treated with 100 mM sodium carbonate pH 11.5 as described (Fujiki et al. 1982; Sam-Yellowe and Ndengele 1993), the second group was treated with 0.5 % sodium dodecyl sulphate (SDS) and both groups were emulsified in Freund's complete adjuvant for the initial immunization intraperitoneally (i. p.). Fifty

to 100 μg of total protein was used for immunization of six CFW mice per antigen. Subsequent immunizations were administered in incomplete Freund's adjuvant i. p. Mice were immunized five times at weekly intervals, blood was collected and pooled from each group of mice for separation of antisera. Gradient fractions and schizont extracts ranging from approximately 5-10μg of proteins were separated on 5-15 % gradient gels, electrophoretically transferred to nitrocellulose paper (NCP) and probed with the rhoptry specific antisera using previously described methods (Sam-Yellowe et al. 1998). Western blotting was performed with the mouse antisera designated Pb01, Pc01 and Py01 specific for sodium carbonate treated rhoptries of *P. berghei*, *P. chabaudi* and *P. yoelii*, respectively. Antisera Pb02, Pc02 and Py02 specific for SDS treated rhoptires of the three rodent *Plasmodium* species were also used for western blotting.

Mouse erythrocyte binding assay:

Mouse blood was collected by cardiac bleeding of AKR mice into citrate phosphate dextrose buffer (CPD) and washed 3 times in RPMI-1640. Spent culture supernatant (SCS) was collected from short term cultures of rodent *Plasmodium* schizont-infected erythrocytes synchronized to approximately 90 % parasitemia on 65 % percoll (Pasvol et al.1978) and cultured in 5 mls of complete medium; RPMI 1640-Hepes, supplemented with 10 % of heat inactivated fetal bovine serum (FBS). Unlabeled spent culture supernatant (0.4 ml) was added to mouse erythrocytes (0.1 ml), resuspended gently and incubated for 30 min at RT. The binding assay was continued as described (Haynes et al. 1988; Perkins and Rocco 1988). Proteins eluted using 0.5 M NaCl (0.08 ml) were analyzed by SDS-PAGE and western blotting using antiserum #686 specific for *P. falciparum* Rhop-3 (Yang et al. 1996).

Isolation of genomic DNA and Polymerase chain reaction (PCR):

Schizont-infected erythrocytes of *Plasmodium chabaudi*, P. *yoelii* and *P. berghei* were lysed with 10 mM Tris pH 8.8 followed by centrifugation at 15,000 rpm (Beckman JA-20 rotor) for 10 min to obtain schizont pellets. The pellets were homogenized in TES buffer (10 mM Tris-HCl pH 7.6, 50 mM EDTA, pH 8.0, 0.1 % SDS) containing 1 mg/ml proteinase K and incubated in a 50°C water bath overnight. Schizont DNA was extracted with phenol/chloroform, precipitated by 95% ethanol, and resuspended in TE buffer (Sambrook et al. 1989). Following analysis on a 0.7 %

agarose gel, the DNA was used as a template for PCR. The following oligonucleotide primer pairs were designed based on the sequences of exon 3 of *P. falciparum* Rhop-3 (Brown and Coppel 1991), cDNA clones Y1412 and Y1821 for *P. yoelii* (Anthony et al. 2000), for use in PCR : Y1412F 5'-*ggatcc*TGTGCCATGAAACATTTTTAT-3' and Y1412R 5'-*gaattc*TTCATTATATTTGGCCATATC-3'; Y1821F 5'-*ggatcc*TGA GAATATAAGAAGTGATGA-3' and Y1821R 5'- *gaattc*GTTTGTTTCATCATCAT TATC-3'; Rhop-3-3F 5'-*ggattc*ATTGTAGTCCTTTTTAGA-3' and Rhop-3-3R 5'-*gaattc*CCAAATATGCTATATGTC-3'. Restriction cleavage sites shown in lower-case italics were added to permit directional cloning. Amplification by PCR was performed in a 50 µl reaction mixture with PCR reaction buffer containing 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3, 10 mM of each dNTP, 10 pmole of each primer, and 1 U of EnzyOne DNA Polymerase (Enzypol Ltd, Denver CO). PCR was performed using the following cycling parameters; one cycle of 8 min at 50°C; 1 cycle of 1 min at 95°C, 30 cycles of 30 s at 95°C, 30s at 45°C, 1 min at 72°C and 1 cycle each of 7 min at 72°C and 30 min at 35°C. PCR products were analyzed on 1% agarose gels, visualized with ethidium bromide and DNA bands were excised, followed by gel purification using the Prep-A-Gene kit (Biorad, Richmond, CA) according to the manufacturer's protocol. The specificity of the PCR was verified with genomic DNA from *P. yoelii* and *P. falciparum*. PCR was also performed without template DNA as a negative control. Gel purified PCR products were treated with Exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Cleveland, OH) at 37°C for 15 min to remove excess dNTPs and primers and at 80°C for 15 min to inactivate the enzymes. The products were incubated with the corresponding forward primers and DYEnamic ET terminator cycle premix (Amersham Pharmacia Biochem, Cleveland, OH) and subjected to the following cycling reaction: 96°C, 30 sec; 50°C, 15 sec; 60°C, 15 sec; 60°C, 1 min for 30 cycles. Reaction products were passed through Sephadex G50 columns (Amershem Pharmacia) and dried in a Speed-Vac centrifuge (Savant Instruments, Inc., Holbrook, NY). The products were denatured at 70°C for 2 min then mixed with formamide loading dye. Samples were loaded on a SEQ 4 x 4 automatic DNA sequencer (Amersham Pharmacia Biotech., Cleveland, OH). DNA sequencing was also performed by Cleveland Genomics, Cleveland, OH. Sequences obtained were verified by BLAST search (Altchul et al. 1990) and analyzed using Sequencher version 4.0 (Gene Codes Corp. Ann Arbor Michigan).

RESULTS AND DISCUSSION

In order to determine the reactivity of isolated rhoptries with Rhop-3 specific antibodies and to confirm that the integrity of the isolated organelles was stable, isolated organelles were sedimented and, fixed for IEM and reacted with antiserum #686 specific for *P. falciparum* Rhop-3. Figure 1 shows the reactivity of isolated rhoptries from *P. falciparum*, *P. yoelii*, *P. berghei* and *P. chabaudi* with antiserum #686 (Yang et al. 1996). Gold particles were observed within the matrix of the isolated rhoptries (Fig.1) in *P. yoelii* (1A), *P. berghei* (1B), *P. chabaudi* (1C) and *P. falciparum* (1D). The results confirmed the immunological cross-reactivity of the Rhop-3 protein across different *Plasmodium* species and demonstrated that isolated organelles were stable and proteins within the organelles retained reactivity for specific antibody.

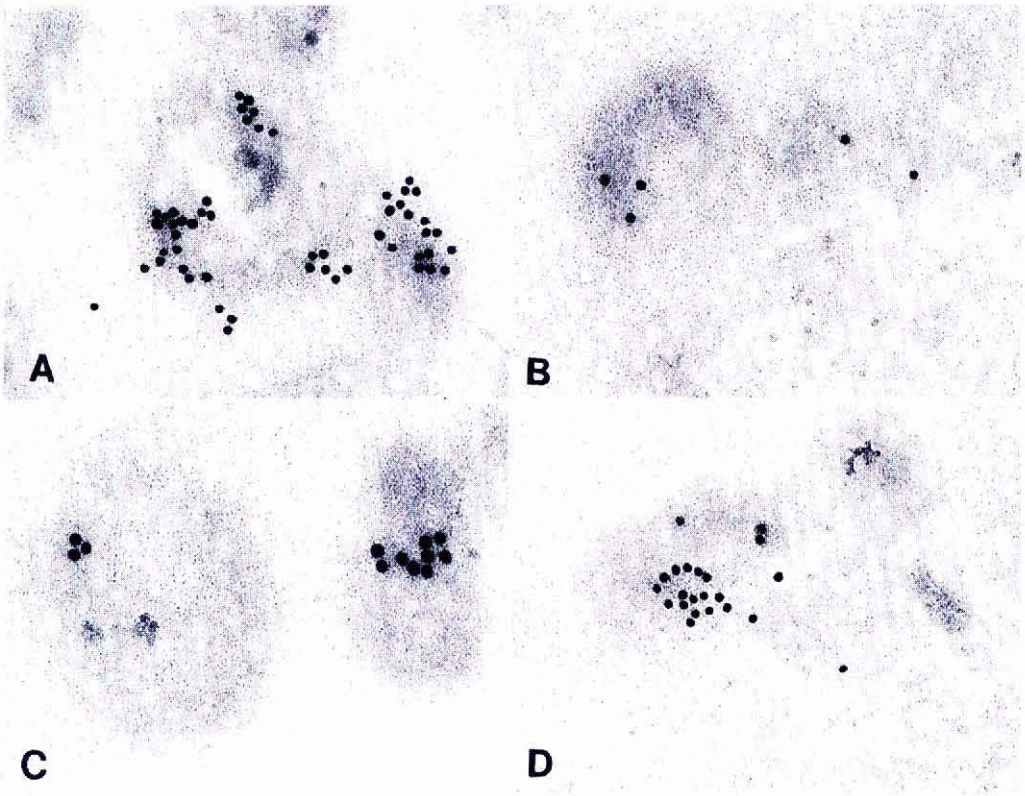

Figure 1. Immunoelectron micrographs of isolated rhoptries of (A) *P. yoelii* (B) *P. berghei* (C) *P. chabaudi* and (D) *P. falciparum* using antiserum #686. Isolated rhoptries were sedimented and processed for IEM using antiserum #686 (Yang et al. 1996). Gold particles (15 nm) indicate the localization of rhoptry protein (Rhop-3) within the isolated rhoptries.

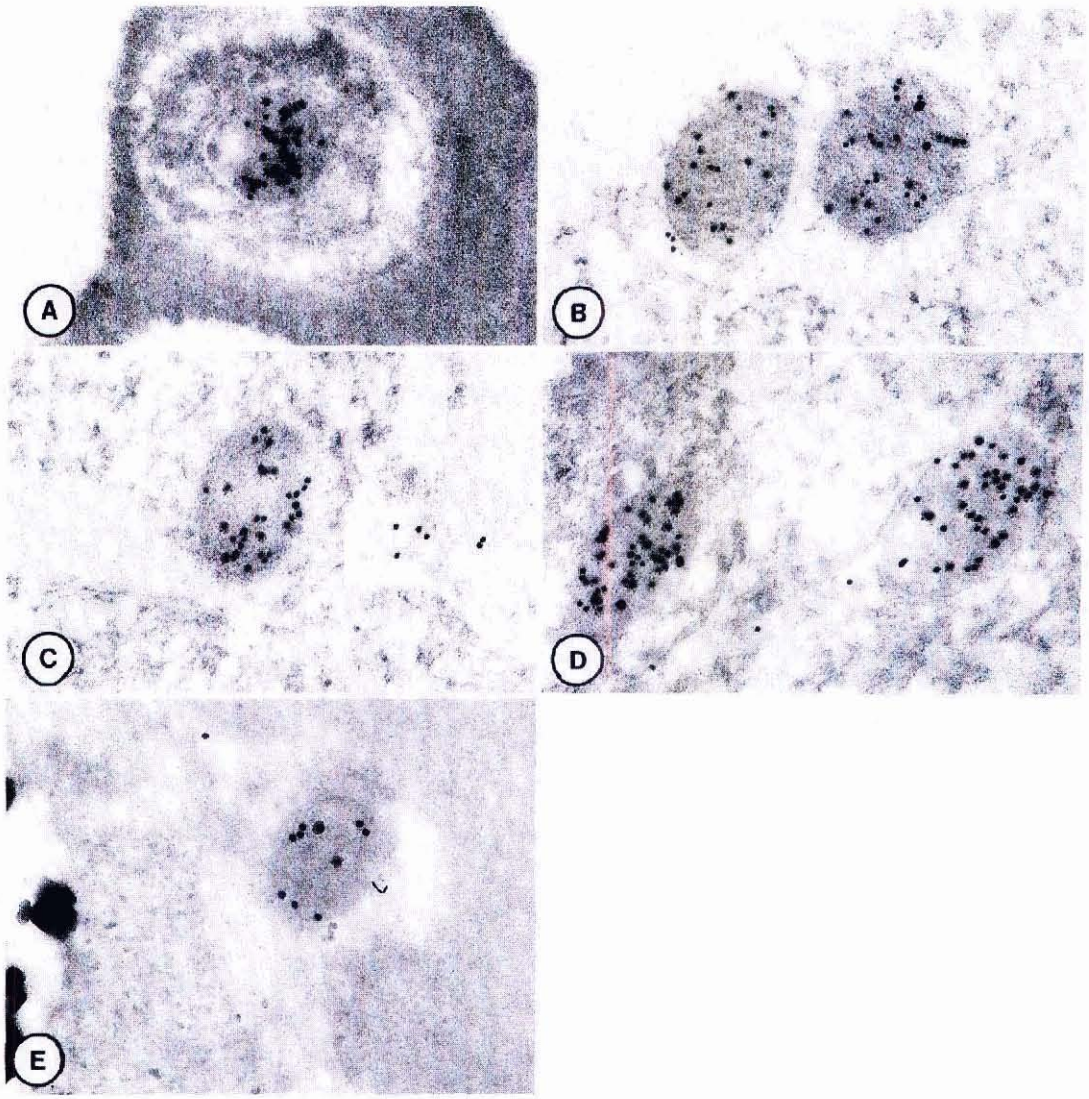

Figure 2. Immunoelectron micrographs of rodent *Plasmodium* rhoptries using rhoptry specific antisera prepared against isolated rhoptries treated with 100mM sodium carbonate, pH 11.5 or 0.5% SDS. (A) *P.yoelii* specific antiserum (Py01) prepared against sodium carbonate treated rhoptries reactive with *P. yoelii* rhoptries; (B) *P. yoelii* specific antiserum (Py02) prepared against SDS treated rhoptries reactive with *P. yoelii* rhoptries; (C) and (D) *P. chabaudi* specific antiserum (Pc01) prepared against sodium carbonate treated rhoptries reactive with *P. chabaudi* rhoptries; and (E) *P. berghei* specific antiserum (Pb01) prepared against sodium carbonate treated rhoptries reactive with *P. berghei* rhoptries. Gold particles (15nm) were observed within the matrix of the rhoptries.

To evaluate the potential for identifying novel rhoptry proteins not accessible when whole untreated rhoptries are used for immunization, rhoptries were subjected to treatment with alkaline sodium carbonate or 0.5 % SDS. Sodium carbonate extracts soluble proteins separating them from integral membrane proteins retained with the pellet following centrifugation. The sodium carbonate treated antigen preparation used for immunization in the current study contained a mixture of both soluble and integral membrane proteins of the rhoptries. Following sodium carbonate treatment, centrifugation was omitted. Sodium dodecyl sulfate denatures proteins by disrupting hydrophobic interactions, leading to the extraction of membrane-associated proteins. The supernatant containing SDS extracts, following centrifugation was used for immunization. The new rhoptry specific antisera were evaluated by indirect immunofluorescence assay (IFA) using fixed segmented schizont infected erythrocytes. A punctate staining pattern indicating staining of the apical complex was obtained (results not shown). Reactivity of the new antisera with rhoptry proteins was confirmed by IEM. Segmented schizont-infected erythrocytes processed for IEM were reacted with the rhoptry specific antisera. The results are shown in Figure 2. The antisera Py01 and Py02 (Fig. 2A and B, respectively) specific for *P. yoelii* rhoptries, treated with sodium carbonate and SDS respectiviely, reacted with *P. yoelii* rhoptries. Antiserum Pc01 specific for sodium carbonate treated rhoptries of *P. chabaudi* (Fig. 2C and D) reacted with *P. chabaudi* rhoptries and antiserum Pb01 specific for sodium carbonate treated *P. berghei* rhotpries (Fig. 2E) reacted with *P. berghei* rhoptries. In the three rodent *Plasmodia*, the matrix of the rhoptries was labeled with gold particles.

The rhoptry specific antisera were also used for Western blotting to detect putative rhoptry proteins in sucrose gradient fractions. Antisera prepared against sodium carbonate and SDS treated rhoptries reacted with sucrose gradient fractions containing isolated rhoptries and recognized protein bands ranging from approximately 20 kDa to 250 kDa, similar to fractions recognized by antiserum #686 specific for the *P. falciparum* Rhop-3 protein, Mabs 25.37 specific for Py235 and 28G2dc1 specific for AMA-1 (Holder and Freeman 1981; Narum and Thomas 1994; Sam-Yellowe et al. 1998, 1999) (Fig. 3, panels A and B). Predominant reactivity was obtained in gradient fractions corresponding to densities of 1.12 – 1.17 g/ml (Fig. 3 A and B, lanes 4-7) as seen previously (Sam-Yellowe et al. 1998). A number of proteins recognized by the antisera were different from those seen previously when antibodies

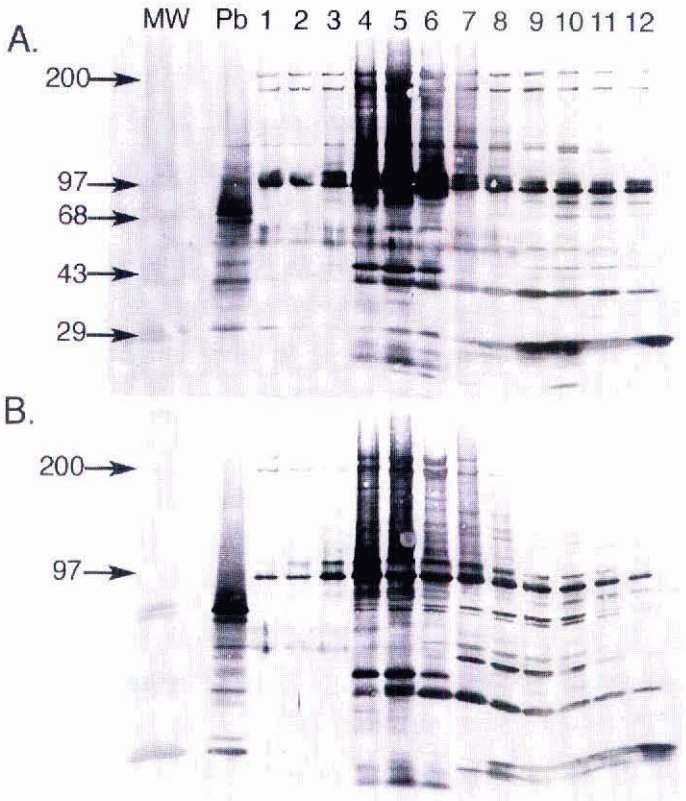

Figure 3. Detection of *Plasmodium berghei* rhoptry-associated proteins in sucrose gradient fractions by western blotting of SDS-PAGE separated proteins transferred to nitrocellulose. (A) *Plasmodium berghei* proteins were probed with *P. berghei* rhoptry specific antiserum Pb01 prepared against sodium carbonate treated rhoptries; (B) *P. berghei* proteins were probed with *P. berghei* rhoptry specific antiserum Pb02 prepared against SDS treated rhoptries. Lanes 1-12 represent individual sucrose gradient fractions collected following ultracentrifugation. Lane Pb was loaded with a *P. berghei* schizont extract. The predominant reactivity of the two antisera was to proteins in lanes 4-7 representing gradient densities in the range of 1.12-1.17 g/ml. Both antisera recognized several proteins of similar molecular weight. Relative MW (molecular weight) was estimated relative to apparent weights of prestained molecular weight standards (GIBCO BRL); myosin, 200 kDa; phosphorylase B, 97kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 29 kDa; beta-lactoglobulin, 18 kDa and lysozyme, 14 kDa.

against whole untreated rhoptries were used in Western blotting (Sam-Yellowe et al. 1998). Additional high molecular weight protein bands not seen with antisera prepared against whole organelles, were observed using the new antisera prepared against sodium carbonate and SDS treated isolated rhoptries. The protein bands were clustered in the range of 100 kDa –250 kDa. The results shown in Figure 3 A and B are for antibodies prepared against sodium carbonate (Pb01) and SDS (Pb02) treated isolated rhoptries of *P. berghei*, respectively. Similar western blotting results were obtained for *P. chabaudi* and *P. yoelii* (results not shown). The results indicate that treatment of the rhoptries with SDS and sodium carbonate led to exposure of rhoptry proteins not easily accessible when whole untreated organelles are used for immunization. The use of the new antisera for identifying additional erythrocyte binding proteins and for isolating rhoptry genes is in progress.

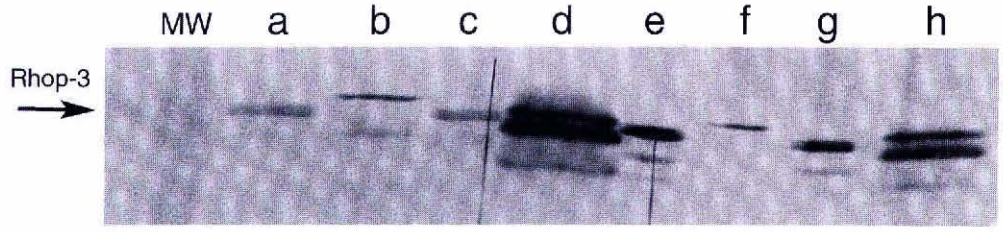

Figure 4. Western blot of rodent *Plasmodium* Rhop-3 bound to and eluted from AKR mouse erythrocytes using 0.5 M sodium chloride. Eluted proteins were reacted with antiserum #686 specific for *P. falciparum* Rhop-3. Lanes (a-d), spent culture supernatants of *P. yoelii* strain 17XL, *P. chabaudi adami*, *P. berghei* strain K-173, and *P. falciparum* strain FCR-3 respectively. Lanes (e-h), Rhop-3 from *P. yoelii* strain 17XL, Rhop-3 from *P. chabaudi adami*, Rhop-3 from *P. berghei* strain K-173 and Rhop-3 from *P. falciparum* strain FCR-F86. Antiserum #686 reacted with the Rhop-3 protein in eluates from rodent *Plasmodia* and *P. falciparum*.

We examined the erythrocyte binding properties of the Rhop-3 protein from the rodent *Plasmodium* species. Spent culture supernatants from short-term cultures of schizont-infected mouse erythrocytes were used in an erythrocyte binding assay (Haynes et al. 1988; Perkins and Rocco 1988; Sam-Yellowe and Perkins 1990). The Rhop-3 protein eluted from mouse erythrocytes following incubation with SCS from the three rodent *Plasmodium* cultures bound to erythrocytes in a manner similar to that shown for *P. falciparum* Rhop-3 (Fig. 4). An approximately 96 kDa/100 kDa protein reacted with the Rhop-3 specific antiserum in SCS of the three rodent *Plasmodium* species (Fig. 4, lanes a-c) and in the NaCl eluates obtained from the

mouse erythrocytes (Fig. 4, lanes e-g). This was similar to results obtained for *P. falciparum* (lanes d and h) for the 110/100 kDa Rhop-3 protein. The Rhop-3 protein in *P. chabaudi* (lanes b and f) appeared as a doublet in some experiments with the top band migrating slower than the Rhop-3 from *P. berghei* and *P. yoelii*. We speculate that there may be size variation within the Rhop-3 protein family. Additional investigations will be required to confirm this observation. The data suggest that the Rhop-3 protein in the different *Plasmodium* species probably plays a similar role during merozoite invasion. Other apical complex proteins of *Plasmodium* species (Ogun and Holder 1996; Sim 1990; Sam-Yellowe and Perkins 1990; 1991; Sam-Yellowe 1992; Kappe et al. 1998) possess erythrocyte binding properties, indicating a shared property for the proteins associated with the invasion process.

Based on our hypothesis that the rodent *Plasmodium* species possess the Rhop-3 gene homologue, we identified the Rhop-3 gene homologue in *P. yoelii* (Anthony et al. 2000). In the present study, we investigated the presence of the Rhop-3 gene homologue in *P. bergehi* and *P. chabaudi* using homology cloning by PCR, DNA sequencing and analysis of the deduced amino acid sequences. Interspecies sequence similarity in the Rhop-3 C-terminus of *P. falciparum*, *P. berghei*, *P. chabaudi* and *P. yoelii* is shown in Figure 5. Agarose gel analysis of PCR products obtained following amplification with exon 3 primers of the Rhop-3 gene showed the amplification of an approximately 980 bp DNA fragment (results not shown). In addition, primers constructed based on *P. yoelii* clone Y1821 (Anthony et al. 2000) amplified a DNA fragment of approximately 1 kb. The amino acid sequence encoded by clone Y1821 showed a greater degree of variation than the region encoded by clone Y1412 in *P. yoelii*. (Anthony et al. 2000). When oligonucleotide primers constructed from clone Y1412 of *P. yoelii* were used for PCR, a DNA fragment of approximately 1.2 kb, corresponding to the expected size, was amplified. BLAST search (Altschul et al. 1990) and deduced amino acid alignment for the Rhop-3 gene from the three rodent *Plasmodium* species with *P. falciparum* Rhop-3 is shown in Figure 5. The results demonstrate the conservation of amino acid sequences in the C-terminus of the four *Plasmodium* species. The overall similarity in the amino acid sequences among the rodent *Plasmodium* species was greater than the similarity to *P. falciparum*. However, since strong immunological cross-reactivity was observed among the Rhop-3 protein from the four species, it appears that the amino acid differences observed do not affect protein folding or conformation. A conserved cysteine residue, seen in all three

rodent *Plasmodium* Rhop-3 and *P. falciparum* Rhop-3 is shown in bold (Fig. 5). The amino acids flanking the cysteine residue, **Y**-C-**H** in the rodent *Plasmodium* Rhop-3 are the same, while they vary in the *P. falciparum* sequence, **S**-C-**F**. The significance of the variation is not known. The region of Rhop-3 analyzed in the present study represents amino acid 570-676 of the *P. falciparum* Rhop-3 protein, a region in the C-terminus that is recognized by human serum from different geographic regions endemic for malaria (Yang et al. 1996). This region was also well conserved among three laboratory strains of *P. falciparum* examined; (HB-3-Honduras, FCR-F86-The Gambia and FCH-17 (Tanzania) and a clinical isolate from Kenya (Sam-Yellowe, T. Y. in preparation). Conservative amino acid substitutions were observed in laboratory isolates of *P. falciparum* (Yang et al. 1986; Sam-Yellowe, T. Y. in preparation). Areas of interspecies variation, although limited, were also observed in the Rhop-3 nucleotide and amino acid sequences. However, the amino acid changes represented areas of synonymous nucleotide mutations suggesting that protein conformation was not affected. The C-terminus of AMA-1 is also highly conserved among *Plasmodium* species and among laboratory and field isolates of *P. falciparum* (Oliveira et al. 1996; Kappe et al. 1998). In addition, the RAP-1 rhoptry protein of *P. falciparum* shows limited sequence variability among *P. falciparum* isolates (Howard and Peterson 1996). Results from other investigators and those reported in the current study underscore the importance of continued investigations regarding the immunological significance of merozoite rhoptry proteins as potential vaccine molecules.

Pc	----------	--LT F*LP-	FNKGTKKFLY	Y – FISIIAIL	HINR –YYYEQ	IYCHHNEHFD
Pb	-------FFEY	SIMDFFTSY	FNKGTKKFIY	Y –FISVISIL	HINR- YYYEQ	IYCHHDKYFE
Py	-------FFEY	SIMDFFTSY	FNKGTKKFIY	Y - FISVISIL	HINR -YYYEQ	IYCHHDKYFE
Pf	SNPSSPFFD -	TI I EFLVTY	YNKGSEKFVL	Y - FISI ISVL	YINE - YYYEQ	LSCFY PKEFE
570						
Pc	ALKSKMIHPD	IVKGIIKKLR	SILNKP - KYSK	M--------	MELYNKLESD	TLFNYDE---
Pb	ALKSKMIHPD	IVNKILEKIK	SILNTP - RYSK	M--------	LE - YNKLEDD	KLFDYDE---
Py	ALKSKMIHPD	IVNKILEKIK	SILNTP - RYSK	M---------	LEL YNKLEDD	KLFDYDEMVK
Pf	L I KSRMIHPN	IVDRILKGID	NLMKSTRYDK	M---------	RTMYLDFE SS	DI-FSRE-KVFT
						676

Figure 5. Deduced amino acid sequence alignment of the C-terminus region of Rhop-3 of *P. falciparum* (Pf), *P. chabaudi adami* (Pc), *P. berghei* (K173) (Pb) and *P. yoelii* (Py). Rodent parasite Rhop-3 sequences were obtained following sequencing of PCR products amplified using primers constructed from *P. yoelii* Rhop-3 (see appendix B8). The numbers denote the numbering of amino acid residues based on *P. falciparum* Rhop-3 (amino acid residues 570-676, Brown and Coppel 1991). The asterisk (*) denotes an internal stop codon identified in the open reading frame (ORF) of Pc. Dashed lines in the alignment were introduced to adjust spacing to give the overall best fit. A conserved cysteine residue is shown in bold.

The presence of the Rhop-3 gene in the rodent *Plasmodium* species, the demonstration that *P. falciparum* merozoites can invade mouse erythrocytes (Klotz et al 1987; Sam-Yellowe and Perkins 1990) and that the Rhop-3 protein is also secreted into mouse erythrocyte membranes during merozoite invasion, suggests that the immunological and biological role of Rhop-3 can be investigated in detail using the rodent *Plasmodium* models. A similar secretion of the dense granule protein RESA (ring-infected erythrocyte surface antigen) into mouse erythrocytes was also described (Klotz et al. 1987) indicating that apical complex proteins play similar roles during merozoite invasion in the different *Plasmodium* species. As summarized in Table 1 and reported in our previous studies invasion levels of *P. falciparum* into mouse erythrocytes can be high depending on the strain of *P. falciparum* examined (Sam-Yellowe 1992), and the strain of mouse used.

Table 1. Invasion of *P. falciparum* into erythrocyte from eight different mouse strain.

Mouse strain	Untreated	Try[a]	Chy[a]	Neuraminidase	α-gp3[b]
BALB/cJ	41±11	8±4	11±1	26±3 (29)	16
AKR/J	55±15	13±1	16±5	24±2 (21)	25
DBA/2J	33±9	17±4	19±7	25±2 (31)	30
DBA/1J	33±8	ND	ND	ND	ND
CBA/J	37±6	ND	ND	ND	ND
CFW	36±15	ND	ND	ND	ND
C57BL6/J	22±3	12±8	10±3	28±9 (27)	13
B10.BR/SgSnJ	26±5	8±4	11±3	24±10 (25)	17
Human erythrocyte	100	6±2	15±13	62±2 (74)	68

Results of invasion are expressed as the mean ± range of two or three separate experiments performed in duplicat. a: Trypsin and chymotrypsin was used at the concentration of 1mg/ml^{-1}. b: Results of a single experiment. Similar inhibition results obtained with different α-gp3 antiserum (1:25). ND: no data. Neuraminidase used (54 mU/ml^{-1}) was from *Clostridium perfringes*. Similar results were obtained with *Vibrio cholerae* neuraminidase (50 mU/ml^{-1}). The invasion rates are shown in parenthesis and represent the results of a single experiment. Neuraminidase hydrolyzes α 2,3-, α 2,6- or α 2,8- terminal N- or O-linked sialic acid residues from glycoproteins, mucopolysaccharides and in various oligosaccharides. Both enzymes share similar specificities.

In our previous studies we have used the mouse erythrocyte as a heterologous model to investigate the role of the Rhop-3 protein during *P. falciparum* merozoite invasion (Sam-Yellowe, et al. 1990, 1991; Sam-Yellowe and Ndengele 1993; Sam-

Yellowe 1993; Ndengele et al. 1995), and early stages of intracellular parasite development. With the demonstration of Rhop-3 in the rodent *Plasmodium* models and the participation of the Rhop-3 protein during invasion, the contribution of the Rhop-3 protein to the development of blood stage immunity can be further investigated.

Apicomplexan rhoptry proteins are highly immunogenic and well conserved in structure (Perkins 1992). In addition, immunization with whole rhoptries was shown to confer protection in *Babesia* sp. (Machado et al. 1999), suggesting the additive protective effects of different rhoptry proteins. The sequence similarity of Rhop-3 genes between *P. falciparum* and the rodent *Plasmodium* species suggests that the Rhop-3 gene product may play a significant role during invasion, underscoring the importance of limited polymorphism in the protein. Based on the data, we conclude that rodent *Plasmodium* species can serve as suitable in vivo models to directly investigate the biological role of Rhop-3 during invasion and intracellular development. The contribution of the Rhop-3 rhoptry protein to the development of blood stage immunity to malaria can be directly investigated in vivo.

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