J. Protozool. Res., 10, 128 - 154 (2000)

Molecular Organization and Cross-linking Analysis of the *Plasmodium falciparum* Erythrocyte Binding Proteins Rhop-H and SERA

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Received May 23, 2000 and Accepted June 26, 2000

Keywords: High molecular weight rhoptry protein complex, Rhoptry protein, erythrocyte binding proteins (EBPs), chemical cross-linking

ABSTRACT

The 120 kDa serine-rich antigen (SERA) of *Plasmodium falciparum* and the high molecular weight rhoptry protein complex (Rhop-H) bind to erythrocytes and are co-eluted by high salt. Their interaction with the host erythrocyte may be of crucial importance to merozoite invasion and parasitophorous vacuole membrane (PVM) formation. We investigated the timing of protein release into the SCS. The release of Rhop-H and SERA into schizont SCS was detected two hours after removal of biosynthetic label with maximum levels detected at twenty-five hours. Optimal protein binding to erythrocytes was obtained with SCS collected at twenty-five hours. Both proteins were detected in the SCS of schizont stages by immunoprecipitation,

but not in ring and trophozoite stages. The identity of SERA was confirmed using different antibodies specific to SERA and no immunological cross-reactivity was detected between SERA and Rhop-H. Affi-blue gel chromatography of SCS for enrichment of both proteins for erythrocyte binding and analysis by immunoblotting showed SERA and Rhop-H in the same fractions. The topological distribution of the proteins on the erythrocyte surface was investigated by chemical cross-linking analysis using DTSSP. SERA and Rhop-H proteins bound at a distance of approximately 12Å on the mouse erythrocyte surface with no other associated proteins. Similar results were obtained using cross-linked schizont extracts precipitated by specific antibodies and analyzed on two-dimensional (2-D) SDS-PAGE gels. Sedimentation analysis demonstrated that SERA and Rhop-H do not form a complex in vivo, and both types of proteins possess different sedimentation rates. We conclude that prior association of SERA with Rhop-H is not required for erythrocyte binding.

INTRODUCTION

Several studies have demonstrated the immunogenicity of *Plasmodium falciparum* Rhop-H and SERA (Barr et al. 1991; Inselberg et al. 1993; Siddiqui et al. 1987). Antibodies against native and recombinant SERA inhibit merozoite invasion in vitro and SERA specific antibodies complexed with native SERA have been identified in in vitro immune complexes following merozoite release (Lyon et al. 1989). The SERA antigen has also been identified in the lumen of the PVM and on the surface of the invading merozoite (Perkins and Zeifer 1994; Li et al. 1989; Delplace et al. 1998). It is the major protein found in the PVM in schizont stages. In addition, the Rhop-H protein complex consisting of proteins of 140/130/110 kDa, also implicated in PVM formation were identified in the erythrocyte membrane following merozoite invasion (Sam-Yellowe et al. 1988). Both groups of proteins bind phospholipids typically located in the inner leaflet of the erythrocyte with SERA showing a preference for short chain fatty acids (Perkins and Zeifer 1994). Both Rhop-H and SERA have been identified in all isolates of *P. falciparum* examined so far (Sam-Yellowe 1993; Gor et al.1998).

Little is known about the association of SERA and Rhop-H during parasite development and during erythrocyte membrane binding. During merozoite release and erythrocyte reinvasion, SERA is processed into a 47 kDa N-terminal domain, a

50 kDa central domain and an 18 kDa C -terminal domain. The 47 kDa domain contains parasite inhibitory epitopes and as such is of considerable interest in vaccine studies (Fox et al.1997; Debrabant et al.1992). Due to the importance of SERA as a vaccine candidate for malaria, the importance of Rhop-H in merozoite invasion and the observation that both proteins are erythrocyte binding proteins, we wanted to investigate the organization and interaction of both groups of proteins. Our objective was to re-examine the erythrocyte binding properties of Rhop-H and SERA in an attempt to characterize in more detail the interaction between the two groups of proteins. The following central questions were investigated in this study: 1) Are the Rhop-H and SERA proteins associated in vivo? 2) Do the Rhop-H and SERA proteins associate once bound on the erythrocyte surface? 3) What other parasite proteins bind in close proximity to Rhop-H and SERA on the erythrocyte surface? In order to address the three central questions of the study, the identity of the proteins and the specificity of the antibodies were verified. In an effort to investigate the mechanism of protein interaction between the two groups of proteins and to understand the biochemical mechanisms by which the interactions occur, we performed different biochemical and immunochemical studies using chemical cross-linking of extracted native parasite proteins and proteins bound to mouse erythrocytes, and sedimentation analysis of both types of proteins. *Plasmodium falciparum* invades mouse erythrocytes and Rhop-H and SERA proteins bind directly to the intact erythrocyte, to membranes and to inside-out vesicles. This mouse erythrocyte model established in previous studies (Sam-Yellowe and Perkins 1990; Klotz et al. 1987) was used in this study to investigate the interactions of Rhop-H and SERA with the erythrocyte surface and the interaction among the parasite proteins. For unequivocal identification of complexed proteins, antibodies highly specific for SERA and Rhop-H were used for immunoprecipitation and immunoblotting analysis.

MATERIALS AND METHODS

In vitro cultivation and metabolic labeling of P. falciparum

Synchronous cultures of 7G8 (Brazil) were maintained in vitro according to the methods of Trager and Jensen (1976). *Plasmodium falciparum* was 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. Labeling with [^{35}S]-methionine

and collection of culture supernatant for erythrocyte binding assays was performed as described (Sam-Yellowe and Perkins 1990).

Stage-specific SCS collection and time-course of protein release was performed to monitor the appearance of the Rhop-H and SERA proteins in the SCS. *Plasmodium falciparum* cultures were doubly synchronized, initially by 5% sorbitol (Pasvol et al. 1978) to obtain ring stage parasites and then by 65% Percoll (Lambros and Vanderberg 1979) when the parasites had matured to the schizont stage. The concentrated schizonts (approximately 80% parasitemia) were divided into two aliquots. One aliquot was biosynthetically labeled with 1.0 mCi [^{35}S]-methionine (0.1 mCi/ml), distributed equally into 5 wells of a 24-well tissue culture plate (2 ml culture/well) and cultured. For time course collection of the SCS from synchronized parasites, label was removed by centrifugation at 4 h, the mature schizonts resuspended in 2 ml of fresh 10% complete medium and the culture continued. SCS was collected at intervals of 2, 4, 6, 8 and 25 h respectively by centrifugation. The SCS collected with the addition of protease inhibitors (Sam-Yellowe 1993) was used in erythrocyte binding assays. The second aliquot was also distributed in five wells but remained unlabeled and served as control cultures. Synchronized schizont-infected cells were also diluted to 1-2% parasitemia with uninfected erythrocytes and distributed in 4 wells of a 24-well microtiter plate. The ring stage parasitemia was approximately 20%. At time points corresponding to rings (6 h), T = 0, trophozoites (22 h), T = 1, early schizonts (32 h), T=2, late schizonts (42 h), T=3 and segmented schizonts (48 h), T=4, [^{35}S]-methionine at 0.25 mCi/ml was added and parasites labeled for 2 h. At the end of the labeling period infected erythrocytes were collected from each well into 15 ml centrifuge tubes, centrifuged for 5 min at 1,500 rpm, the SCS collected and a protease inhibitor cocktail (Sam-Yellowe 1993) added and the SCS stored at –70°C until used for binding studies. The infected erythrocyte pellets were lysed with 10 mM Tris pH 8.8 and the pellets obtained also stored at –70°C. Smears for Giemsa staining, fixed in absolute methanol and for IFA, fixed in ice-cold acetone were prepared from the unlabeled cultures to monitor parasite development.

Monoclonal and polyclonal antibodies

A summary of the different antibodies used in this study is shown in Table 1. Due to the differences in epitope recognition in a given biochemical or immunochemical experiment, several antibodies specific for Rhop-H and SERA were

used to unequivocally detect and confirm the identity of proteins. Eleven monoclonal antibodies prepared against the 47 kDa N-terminal fragment of SERA were used. The antibodies were prepared against the following two peptides; YIDVDTEDTNIELRT TLKKT-fusion 151 and FESNSGSLEKKKYVKLPSNG-fusion 152. Both sequences were derived from the p126 (SERA) protein from the CAMP strain of *P. falciparum*. The antibodies were checked for reactivity to SERA by immunoblotting, immunoprecipitation and immunofluorescence (IFA). The antibodies were also used to detect SERA in erythrocyte binding assays. All clones were positive in the assays listed. Mab 152.3F7.1.1 (fusion 152) was selected for more detailed characterization due to its stronger reactivity. A monoclonal antibody 5E3 specific for SERA (Lyon et al 1989) was also used. In addition, antibodies against a vaccinia recombinant vP870 (generously provided by Dr. Enzo Paoletti, Virogenetics Corp., Troy NY) (Tine et al. 1996) was also used. Polyclonal antibody against SERA 1 (amino acids 24-285 of the complete SERA molecule of 989 amino acids, generously provided by Dr. Joseph Inselburg, Dartmouth, Medical School, Hanover, NH), antiserum #685 was produced in a New Zealand White rabbit by intrasplenic deposition (Nilsson et al. 1987) of SERA 1 antigen (20 μg) (see Table 1). SERA1 was dissolved in PBS containing 0.05% SDS. For immunization, anesthesia was induced in the rabbit by an intramuscular injection of a cocktail of 5 ml ketamine (100 mg/ml) (Fort Dodge Animal House, Fort Dodge, IA), 5 ml xylazine (20 mg/ml) (Boeringer Ingelheim Vetmedica Inc., St. Joseph's, MO) and 1 ml Acepromazine® (10 mg/ml) (Boeringer Ingelheim) The dose was 1 ml/kg body weight. The surgical site was prepared by clipping, scrubbing with Betadine® surgical scrub and an application of Betadine® solution. Asepsis was practiced throughout the procedure. A midline incision was made beginning at the xiphoid and continuing it approximately 3 cm posteriorly. The spleen was exposed and immobilized. A small slit was made in the capsule and two antigen impregnated sterile nitrocellulose paper discs, each containing 10 μg SERA 1 in 0.05% SDS, 1 mM EDTA in 1 X PBS were placed beneath the capsule. The slit was closed with a single suture of 4-0 plain gut. The peritoneum and muscle layers were closed with simple interrupted sutures of 2-0 Vicryl®. The skin was closed with simple interrupted sutures of 2-0 braided stainless steel wire. Booster injections were administered intravenously (i.v.) at two to three week intervals. Blood was collected before each boost for serum collection. The following Rhop-H specific antibodies were used in this study: Mab 1B9 specific for the 110 kDa rhoptry

protein Rhop-3 coprecipitates Rhop-1/140 kDa and Rhop-2/130 kDa (Sam-Yellowe et al. 1988. Antiserum 680 (Sam-Yellowe et al. 1995) prepared against the Rhop-H complex and specific for the three proteins was used, antiserum 686 (Yang et al. 1996) specific for a Rhop-3 recombinant protein RH3 3-5 was used and antiserum K15 (generously provided by Dr. George S. N. Hui, University of Hawaii, Honolulu Hawaii) specific for the Rhop-H complex was also used (summarized in Table 1).

Table 1. Summary of antibodies used in the study.

Antibodies	Specificity	References
151.1F2.2.2	47 kDa N terminus SERA fragment	current study
151.2F2.1.1	47 kDa N terminus SERA fragment	current study
151.2F8.1.2	47 kDa N terminus SERA fragment	current study
151.4F7.4.1	47 kDa N terminus SERA fragment	current study
151.7D6.4.1	47 kDa N terminus SERA fragment	current study
151.12G9.1.1	47 kDa N terminus SERA fragment	current study
152.2A10.3.1	47 kDa N terminus SERA fragment	current study
152.3D1.1.1	47 kDa N terminus SERA fragment	current study
152.3D3.2.1	47 kDa N terminus SERA fragment	current study
152.3F7.1.1	47 kDa N terminus SERA fragment	current study
152.5E6.1.1	47 kDa N terminus SERA fragment	current study
5E3	SERA	Lyon et al. 1989
vP870	vaccinia recombinant	Tine et al. 1996
#685	SERA 1	current study
1B9	Rhop-H*	Sam-Yellowe et al. 1988
#680	Rhop-H	Sam-Yellowe et al. 1995
#686	Rhop-3 recombinant	Yang et al. 1996
K15**	Rhop-H	

* Mab 1B9 specific for the Rhop-3/110 kDa rhoptry protein of *P. falciparum* coprecipitates Rhop-H.

** Antiserum K15 was generously provided by Dr. George S. N. Hui, University of Hawaii, Honolulu, Hawaii.

Immunofluorescence and Immunoelectron microscopy

For IFA, thin smears of *P. falciparum* schizont-infected erythrocytes were prepared, fixed in acetone for 5 min at 4°C and incubated with SERA specific

antibodies as described (Sam-Yellowe et al. 1988). Smears mounted with 50% glycerol in PBS were visualized by ultraviolet (UV) fluorescence microscopy on a Nikon Labophot microscope. For IEM *P. falciparum* segmented schizonts fixed for 30 min at 4°C with 1% formaldehyde, 0.1% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4 were washed, dehydrated and embedded in LR White resin (Polysciences Inc., Warrington, PA) as described (Yang et al. 1996). Thin sections were incubated with Mab 152.3F7.1.1 and antiserum # 685 diluted 1:10. Negative controls included pre-immune rabbit serum or PBT applied as primary antibody. Following primary antibody incubation, 15 nm gold-labeled goat anti-mouse IgG (Amersham Life Science, Arlington), diluted 1:20 in PBT was applied. Samples were examined in a Zeiss CEM902 electron microscope (Zeiss, Oberkochen, Germany).

Erythrocyte binding assay

Mouse and human erythrocyte binding assays were performed as previously described (Sam-Yellowe and Perkins 1990). Briefly BALB/c mouse erythrocytes collected by cardiac bleeding and human erythrocytes were incubated with biosynthetically labeled SCS. Two hundred-500 µl (100 µCi) of SCS collected from synchronized segmented schizont cultures at 80-90% parasitemia were used for incubation with 100 µl of erythrocytes (1 x 10^9 cells/ml) per eppendorf tube. Binding was carried out at RT for 1 h, the erythrocytes were washed with 500 µl of ice cold RPMI over a second oil gradient, centrifuged and bound proteins eluted by adding 80 µl of 0.5 M NaCl to the erythrocyte pellet per tube and incubating for 15 min at RT. In previous experiments, incubation times of 37°C, RT and 4°C were investigated for protein binding and RT found to be optimal (Sam-Yellowe and Perkins 1990). In addition, a range of 0.2-1 M NaCl was used for protein elution at varying incubation times (0-30 min), and 0.5 M at 15 min found to be optimal. Eluted proteins were analyzed by SDS-PAGE and fluorography. Immuoprecipitation of eluted proteins was performed using Rhop-H and SERA specific antisera previously complexed to goat anti-mouse IgG (Cappel) or protein A-Sepharose (Pharmacia).

Immunoprecipitation and immunodepletion assays

Immunoprecipitation of biosynthetically labelled stage specific parasites and eluted proteins was essentially as described (Sam-Yellowe et al. 1988; Sam-Yellowe and Perkins 1990). Briefly stage specific parasites metabolically labeled with [^{35}S]-

methionine (0.5-1mCi/ml) were extracted with 1 % NP-40/0.1% DOC (1% Nonidet-P40/0.1% sodium deoxycholate in PBS). Aliquots (0.1 ml) of the extract were incubated with goat anti-mouse IgG sepharose (0.03ml) or protein A-Sepharose previously complexed separately with Mabs 1B9 specific for the 110/100 kDa Rhop-3 protein (Sam-Yellowe et al. 1988), or rabbit antiserum #685 specific for SERA, respectively. Pre-immune rabbit serum was used as negative control. Immune complexes were solubilized by boiling for 2 min in 0.05 ml of electrophoresis sample buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 0.001% bromophenol blue) with 100 mM DTT for SDS-PAGE and fluorography.

Immunodepletion experiments were performed to investigate immunological cross reactivity of SERA and Rhop-H specific antibodies. Spent culture supernatant (500 µl at 100 µCi/ml) from biosynthetically labeled schizont-infected erythrocytes collected as described (Sam-Yellowe and Perkins 1990; Sam-Yellowe et al. 1995) and Rhop-H and SERA proteins bound to and eluted from mouse erythrocytes (see mouse binding assay), were incubated with goat anti-mouse IgG-Sepharose beads (Cappel) previously complexed with Mabs 1B9 (Sam-Yellowe et al. 1988) or 5E3 (Lyons et al. 1989) specific for Rhop-3/110 kDa and SERA proteins respectively. Mab 1B9 coprecipitates Rhop-1/140 kDa and Rhop-2/130 kDa (Sam-Yellowe et al. 1988). The mouse eluate was diluted 1:2 in ice cold distilled water before incubation with sepharose beads. Three successive incubations with the beads were carried out using the SCS or mouse eluate, for 15 min each incubation at RT. After each incubation, the beads were sedimented by centrifugation in a microfuge for 2 min at 14,000 rpm, and the supernatant was transferred to a fresh tube containing goat anti-mouse sepharose-IgG. Following the third incubation the flow-through was collected for analysis and the beads washed as described (Sam-Yellowe et al. 1988). The immune complexes from each successive immunoprecipitation, the starting material SCS, mouse eluate and the flow through were separated on a 5-15% SDS-PAGE gel and analyzed by fluorography.

Affi-blue gel chromatography for enrichment of parasite proteins

In order to enrich for SERA and Rhop-H from SCS for the purpose of protein purification and erythrocyte binding unlabeled SCS (5 ml containing 12-16 mg/ml total protein) was loaded on an Affi-blue gel (Biorad, Hercules, CA) chromatography column. Serum proteins such as albumin would be removed and any protein

associations detected upon protein elution. The Affi-blue gel chromatography column was prepared by packing 5 ml of Affi-blue beads (Biorad) into a 6 cm column, and equilibrating with PBS. Parasite SCS was loaded onto the column and binding allowed to occur for 45 min at 4°C. Twelve fractions of the flow through were collected, followed by washing with PBS to remove unbound proteins. Bound proteins were eluted with 1 M NaCl and collected in twelve fractions of 2.5 ml each. Following a second wash in PBS, albumin was eluted with 0.5 M sodium thiocyanate (NaCNS) and samples collected also in twelve fractions of 2.5 ml each. The fractions were stored at –70°C, lyophilized and reconstituted in 0.5 ml PBS. Fractions were analyzed for the presence of the Rhop-H complex and SERA by SDS-PAGE electrophoresis, Coomassie blue staining and western blotting with Mabs 1B9, 5E3 and a polyclonal antibody specific for the Rhop-H complex, K15. Protein determination was performed using the Biorad assay. Due to the reduced levels of protein recovery for Rhop-H only SERA was further purified. Fractions 1-7 containing SERA were pooled, separated on a 5-15% gradient gel, and stained in 1% Coomassie blue in distilled water. A band corresponding to the 120 kDa/SERA protein recognized by Mab 5E3 from both ends of the same gel that was transferred to nitrocellulose paper, was excised from the acrylamide gel and the protein electroeluted as previously described (Sam-Yellowe et al. 1995) for use in binding studies. The purified protein was analyzed by silver staining and western blotting. Protein determination to quantitate protein recovery was performed using the Biorad assay.

Chemical cross-linking of Rhop-H and SERA proteins on mouse erythrocytes

In order to investigate the topological distribution of bound proteins on the erythrocyte surface, chemical cross-linking analysis was performed. *Plasmodium falciparum* SCS (200 µl) biosynthetically labeled or unlabeled was incubated with 100 µl (1 x 10^9cells/ml) mouse erythrocytes for 1 h at RT. The cell suspension was layered on a 0.5 ml silicone oil gradient followed by centrifugation at 14,000*g* for 2 min (Sam-Yellowe and Perkins 1990). The unbound supernatant was collected and the oil discarded. The cell pellet was washed with 0.5 ml of ice-cold RPMI by layering and centrifugation on a silicone oil gradient. Bound proteins on the surface of mouse erythrocytes were cross-linked with dimethylsulfoxide (DMSO) or water-soluble homobifunctional cross-linking reagents (Pierce, Rockford, IL), dithiobis

(succiniimidylproprionate) (DSP)-12 Å (DMSO soluble), dithiobis (sulfosuccinimidylproprionate) (DTSSP)-12 Å (water-soluble), disulfosuccinimidyl tartarate (DST)-6.4 Å (water soluble) and ethylene glycol bis (sulfocuccinimidylsuccinate) (sulfo-EGS)-16 Å for 7 min at RT. For determination of optimal concentration of the cross-linking reagents, concentrations of 2.5 mM, 5 mM and 10 mM in PBS were evaluated, and 5 mM found to be optimal. This concentration was used for all subsequent experiments. The cross-linking reaction was terminated by adding 0.1 M glycine, 1% BSA in PBS. Bound and cross-linked proteins were eluted with 0.5 M NaCl or solubilized with 1%, 0.5% or 0.25% Triton X-100 in PBS. Solubilized or eluted proteins were analyzed by immunoprecipitation (Sam-Yellowe et al. 1988) with Rhop-H or SERA specific antibodies, on SDS-PAGE gels under reducing or non-reducing conditions followed by fluorography. Cells incubated with proteins without cross-linking or detergent alone served as controls. Cells without bound protein, treated or untreated with cross-linkers were also used as controls.

Chemical cross-linking of Rhop-H and SERA followed by two-dimensional (2-D) SDS-PAGE

In order to confirm that the cross-linked protein complexes contained SERA and Rhop-H and to determine the nature of their association, schizont extracts were prepared in TET buffer (10 mM Tris-HCl pH 7.5, 20 mM EDTA, 1% Triton X-100), as described (Howard and Reese 1990) and used for cross-linking experiments. Proteins were cross-linked with 5 mM DTSSP by incubating on ice for 30 min. The reaction was terminated by the addition of 0. 1 M glycine-HCl, pH 10 followed by incubating for an additional 30 min on ice. The cross-linked proteins were immunoprecipitated with Mabs 1B9, 152.3F7.1.1 and rabbit antisera vP870, #680, #685 and #686 using goat anti-mouse IgG sepharose or Protein A sepharose as described (Sam-Yellowe et al. 1988) and immune complexes solubilized in electrophoresis buffer and separated on a 5% SDS-PAGE gel in the first dimension at 50 mA for 3 h. Entire lanes containing separated proteins from 5% gels were carefully cut into strips the width of the lane, using a clean razor blade, incubated in electrophoresis sample buffer containing 50 mM DTT for 1 hr at RT and the strips layered horizontally on an 8% preparative SDS-PAGE gel (Ozawa and Kemler 1992). Electrophoresis was carried out in the second dimension at 50 mA for 3 h.

Sedimentation analysis

In order to evaluate complex formation between Rhop-H and SERA sedimentation analysis of proteins was performed. Biosynthetically labeled or unlabeled schizonts were extracted in TET buffer by incubation on ice for 30 min, followed by sonication in a Branson sonicator for 30 s. The suspension was centrifuged in an airfuge (Beckman) for 30 min at 90,000 rpm. The supernatant was collected, pooled and diluted with TET buffer and layered on a 5-20% continuous sucrose gradient. The gradient was centrifuged at 37,552 rpm for 14 hr at 4°C. Fractions were collected by carefully puncturing the bottoms of the gradient tubes with an 18 G needle and collecting twelve fractions of 325 µl per tube. The fractions were diluted with TET buffer and incubated with Mab 1B9 or rabbit antiserum vP870 previously complexed with goat-antimouse IgG and protein A sepharose respectively. Immunoprecipitation was performed as described (Sam-Yellowe et al. 1988). For unlabeled extracts, fractions were lyophilized and mixed with electrophoresis sample buffer. Fractions separated on SDS-PAGE were analyzed by western blotting using SERA and Rhop-3 specific antibodies. High molecular weight protein standards Myosin (H-chain), 200 kDa, phosphorylase B, 97.4 kDa, Bovine serum albumin, 68 kDa, ovalbumin, 43kDa, carbonic anhydrase, 29 kDa, beta-lactoglobulin, 18.4 kDa and lysozyme, 14.3 kDa, (Gibco BRL) were sedimented in parallel gradients and the fractions collected, analyzed by SDS-PAGE and Coomassie blue staining.

For SDS-PAGE and fluorography of biosynthetically labeled samples in all experiments, the specific activity of samples loaded per lane was in the range of 30-80 µCi. For unlabeled parasite proteins analyzed by SDS-PAGE and western blotting, protein concentrations loaded per lane of gels were in the range of 60-90 µg of protein.

RESULTS AND DISCUSSION

The 120 kDa SERA and Rhop-H proteins are recognized by specific antibodies and do not share cross-reactive epitopes

Plasmodium falciparum erythrocyte binding proteins originating from the apical complex play an essential role during merozoite invasion and PVM formation. It is conceivable that the apical complex proteins function in association with other parasite proteins such as the merozoite surface proteins, MSP-1 and the S-antigen and other proteins located in the PV such as SERA, EXP-1 and -2 (Perkins and Rocco

1990; Gunther et al. 1991). The SERA protein is a candidate antigen for vaccine development against malaria caused by *P. falciparum* (Inselburg et al. 1993; Li et al. 1989; Delplace et al. 1998). A number of investigators have described proteins with similar structure and homology to SERA, but with differences in the molecular weight of the proteins. In addition, SERA is thought to possibly function as a cysteine protease (Gor et al. 1998; Knap et al. 1991).

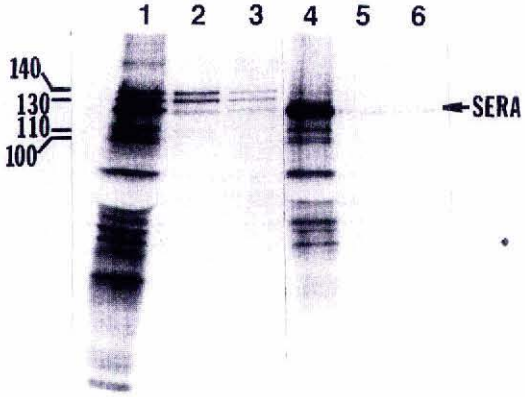

Figure 1A. Mouse erythrocyte binding assay. [^{35}S] methionine labeled *P. falciparum* Rhop-H and SERA proteins were incubated with mouse erythrocytes and bound proteins eluted with 0.5 M NaCl. Lane1, Starting material (SM); 2, Rhop-H bound to and eluted from mouse erythrocytes; 3, Rhop-H eluted from mouse erythrocytes and immunoprecipitated with Mab 1B9; 4 SM immunoprecipitated with Mab 5E3; 5, SERA bound to and eluted from mouse erythrocytes ;6, SERA eluted from mouse erythrocytes and immunoprecipitated with Mab 5E3.

Figure 1B. Immunodepletion of Rhop-H and SERA from SCS and NaCl eluates from mouse erythrocytes. Biosynthetically labeled SCS or NaCl eluates from mouse erythrocytes were sequentially incubated with Mab 1B9 or Mab 5E3 previously incubated with goat anti-mouse-sepharose beads. Immune complexes and the flow-through (FT) were analyzed by SDS-PAGE and fluorography. The starting material (SM) SCS is shown, lane a, mouse eluate; b-d, Rhop-H immunoprecipitated by Mab 1B9 from SM; lane e, F-T after Rhop-H depletion; f-h, Rhop-H immunoprecipitated by Mab 1B9 from NaCl eluates; i, F-T after Rhop-H depletion; j-l, SERA immunoprecipitated by Mab 5E3 from SM; m, F-T following SERA depletion from SM; n-p, SERA immunoprecipitated by Mab 5E3 from NaCl eluates; q, F-T following depletion of SERA from NaCl eluate.

Our aim in the present study was to investigate the nature of protein association between the high molecular weight rhoptry proteins of *P. falciparum*, Rhop-H and the 120 kDa erythrocyte binding protein, SERA. The identity of the individual parasite proteins bound to and eluted from mouse erythrocyte were confirmed by incubating eluates with Rhop-H and SERA specific antibodies. The results in Figure 1A, show that eluted Rhop-H (lane 2) and SERA (lane 5) are precipitated by Rhop-H and SERA specific antibodies (lanes 3 and 6, respectiviely). Antibodies prepared to native and recombinant SERA, SERA 1 and monoclonal antibodies prepared against the 47 kDa immunogenic N-terminal domain were used in this study and each reacted with the 120 kDa protein which we confirmed as SERA. The Rhop-H and SERA proteins bind to the erythrocyte surface and are coeluted by 0.5 M NaCl. However, SERA and Rhop-H proteins do not share cross reative epitopes as demonstrated by immunodepletion and immunoprecipitation experiments using antibodies specific for each type of protein (Figure 1B). Mab 1B9 reacting with Rhop-H depleted Rhop-H proteins in SCS and in eluates recovered from the mouse erythrocyte surface (Fig. 1B, lanes a-d and f-g, respectiviely). The flow-through following Rhop-H depletion contained predominantly SERA (Fig. 1B, lane e). Similarly, following depletion of SERA from SCS and NaCl eluates (Fig.1B, lanes j-l and n-p, respectiviely), the Rhop-H proteins were the predominant proteins remaining in the flow-through of SCS and eluates (Fig. 1B, lanes m and q). The use of specific antibodies in immunoprecipitation of SCS and eluates before and after protein depletion confirmed the lack of cross reactivity of proteins contained in the samples.

SERA is localized to the lumen of the PVM, and both Rhop-H and SERA proteins are released into the SCS in the schizont stage during merozoite release

We studied stage specific protein release into the SCS using highly synchronized parasite cultures. We also investigated the timing of protein release into the SCS. Parasitemia was maintained at approximately 20% following dilution of synchronized segmented schizonts. Fig. 2 shows that the SERA antigen and Rhop-H were not present in the SCS of ring (R) and trophozoite (T) stages, only in mature schizonts (S) and segmented schizonts (Seg). Both groups of proteins were released into the SCS accompanying schizont burst and merozoite release (Fig. 2). The amounts of protein present in the SCS of schizonts before merozoite release probably represents release of proteins from a small percentage of rupturing schizonts (< 0.5%) as observed by Giemsa staining of smears from the culture. Optimal binding of Rhop-

H and SERA proteins to human and mouse erythrocytes was seen with SCS collected at 25 h after the removal of label (Fig. 2). The proteins identified binding to mouse and human erythrocytes were verified to be Rhop-H and SERA by immunoprecipitation with specific antibodies (results not shown). The Rhop-H and SERA proteins bind directly to mouse erythrocytes while MSP-1 and EBA-175 bind directly to human erythrocytes as shown previously (Perkins and Rocco 1988; Sim 1995). In previous studies, we have established the mouse erythrocyte as a heterologous erythrocyte model for use in investigating the role of Rhop-H and SERA during merozoite invasion. Mouse erythrocytes are invaded by different strains of *P. falciparum* in vitro with the secretion of the Rhop-3 and RESA proteins into the mouse erythrocyte membrane (Sam-Yellowe and Perkins 1990; Sam-Yellowe 1992; Klotz et al. 1987). We have obtained similar protein binding results using human erythrocytes perturbed with MSP-1 derived peptides and sublytic concentrations of detergents and membrane modulating agents (Sam-Yellowe and Perkins 1991; Ndengele et al. 1995).

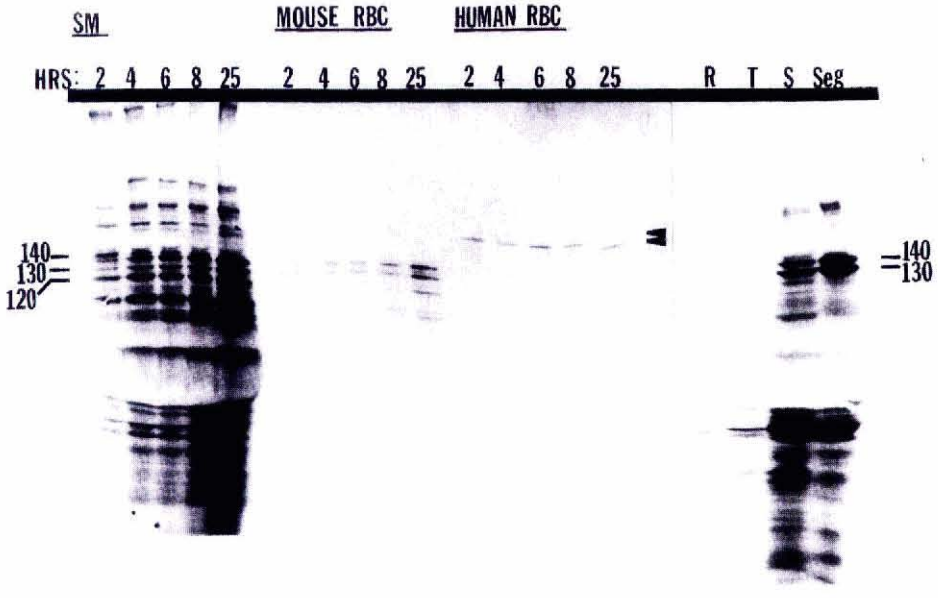

Figure 2. Kinetics of biosynthetically labeled Rhop-H and SERA secretion into SCS of segmented schizonts. Mature schizonts synchronized on 65% Percoll to >90% parasitemia were labeled with [^{35}S] methionine for 4 h, label was removed and SCS collected at 2, 4, 6, 8, and 25 h. The SCS collected after centrifugation was used in binding assays of intact human and mouse erythrocytes. Optimal binding of Rhop-H and SERA to mouse erythrocytes was obtained with SCS collected at 25 h. The two proteins indicated were shown to be MSP-1 and EBA-175 in other studies [35,36] and SERA were seen binding to human erythrocytes. In stage specific experiments to determine the timing of exoantigen secretion into synchronized cultures, predominant protein secretion is seen in the schizont (S) and segmented schizont stage (Seg).

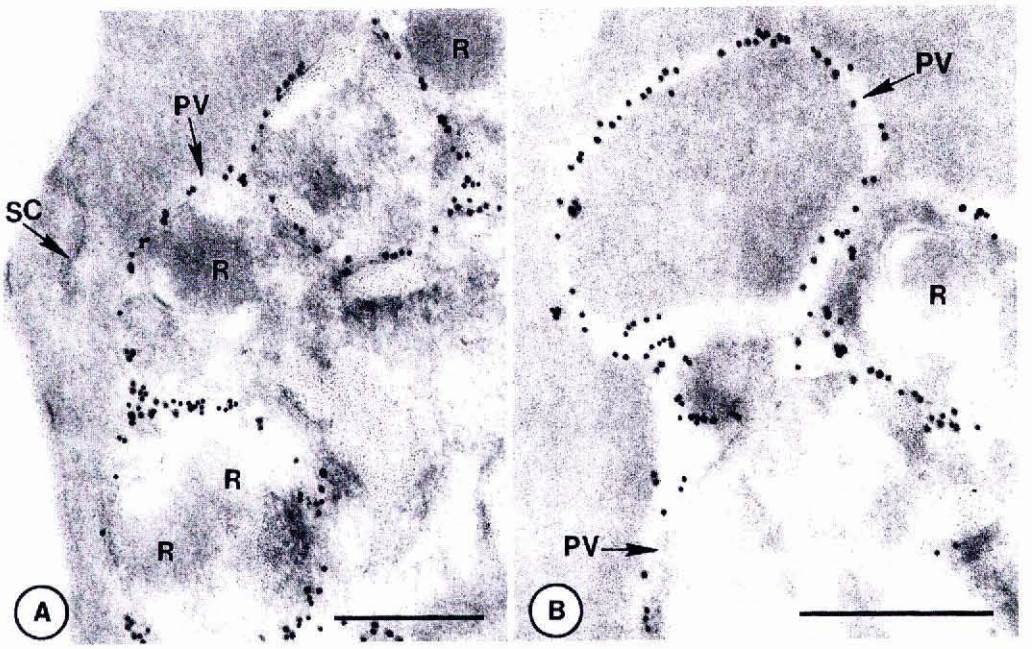

Figure 3. Immunodetection of SERA antigen with antiserum 685 using immunoelectron microscopy. A, SERA is localized to the lumen of the PV. PV=parasitophorous vacuole, R= rhoptries, SC = short cleft. B, Extensions of the PV showing reactivity of the antiserum with SERA. No cross reactivity was seen with the rhoptries and the SC. Bar = 1 μm

In order to confirm the location of SERA with the new specific antibodies generated in the current study, highly specific antibodies produced by intrasplenic deposition of purified SERA 1 and Mab specific for the 47kDa N-terminal fragment were used for immunodetection by IEM. Rabbit antiserum, #685 specific for SERA 1, reacted with SERA in the lumen of the PV and PV extensions (arrows) (Fig. 3 A and B). No cross reactivity was obtained with the rhoptries or short clefts using this antiserum. Mab 152.3F7.1.1 also gave similar reactivity (results not shown). Both SERA specific antibodies also reacted with SERA bound to and eluted from erythrocytes (results not shown).

Rhop-H and SERA proteins bind receptors on the mouse erythrocyte surface at a distance of 12 Å *and are not coprecipitated by specific antibodies*

To enrich for parasite proteins for the purpose of purifying Rhop-H and SERA from the SCS and binding to mouse erythrocytes, Affi-blue gel chromatography was used. SERA and Rhop-H proteins were eluted in the same fractions with 1 M NaCl

and were detected by antiserum K15 (results not shown) and Mab 1B9 reacting with the Rhop-3 protein and with Mab 5E3 specific for SERA (Lyon et al. 1989). In Fig. 4, the results of western blotting with Mabs 1B9 and 5E3 are shown. SERA was eluted in fractions 2-4 with a small amount of protein seen in fraction 5. Similarly Rhop-3 was seen in fractions 2 and 3. The SERA protein enriched, and gel purified from SDS-PAGE gels retained binding properties to erythrocytes. The purified protein bound to erythrocytes and was recognized by specific antibodies (results not shown), suggesting that SERA bound to specific receptors on the erythrocyte independently of Rhop-H. We investigated the topological distribution of both groups of proteins on the erythrocyte surface using membrane impermeant chemical cross-linkers with arm-lengths ranging from 6.4 to 16 Å. We wanted to establish whether Rhop-H and SERA are "nearest neighbors" on the mouse erythrocyte and also if they are associated on the erythrocyte surface when bound. The results shown in Fig. 5 demonstrate that both groups of proteins bind at a distance of approximately 12 Å from each other, they formed a cross-linked complex of >300 kDa seen in unreduced cross-linked samples (Fig. 5, lane 3) which was resolved into the individual components of Rhop-H, Rhop-1/140 kDa, Rhop-2/130 kDa, Rhop-3/110 kDa and SERA/120 kDa following reduction by DTT (Fig. 5, lane 4, see arrows). The distance of 12 Å between proteins demonstrates a close spatial association. No cross-linking was observed with arm lengths of 6.4 or 16 Å (results not shown) and no additional parasite proteins were cross-linked. DTSSP reacts covalently with amino groups on the proteins. The results demonstrate that Rhop-H and SERA are near neighbors and become cross-linked when bound on the erythrocyte surface, forming a heterooligomeric complex. However, each protein appears to interact with an independent erythrocyte receptor in very close proximity, which accounts for the lack of co-precipitation by specific antibodies and the differences in erythrocyte binding observed on enzyme treated erythrocytes (Sam-Yellowe and Perkins 1990). In experiments to confirm the identity of cross-linked proteins in NaCl eluates using specific antibodies for immunoprecipitation, protein signals were weak or absent following fluorography suggesting that antigen epitopes were probably affected by the cross-linkers. Alternatively, cross-linking may have masked epitopes by a change in protein conformation. In alternate cross-linking experiments (discussed below), we demonstrated that the cross-linked proteins are indeed Rhop-H and SERA using specific immunoprecipitation with specific antibodies. In previous studies we showed

that the erythrocyte binding and antibody binding domains of the Rhop-H proteins are distinct (Sam-Yellowe 1993), allowing for the possibility of antibody binding sites to be exposed and affected by the cross-linker. Immunoprecipitation and immunoblotting of cross-linked proteins have been used in other studies to demonstrate evidence of protein association (Ozawa and Kemler 1992; Perkins and Rocco 1990; Ali et al. 1997; Murray and Ohlendieck 1997; Howard and Reese 1990). Moreover, in cross-linking studies of merozoite surface proteins, SERA was cross-linked with MSP-1 and the 130 kDa glycophorin binding protein (GBP-130), but was not detected by antibodies when the complex was analyzed possibly due to the cross-linker used (Perkins and Rocco 1990). We currently do not have evidence to confirm the loss of antibody binding by specific epitopes. However, in studies by other investigators alterations in protein epitopes following cross-linking resulted in loss of reactivity within antibody binding domains (Perkins and Rocco 1990; Murray and Ohlendieck). Control binding experiments without cross-linkers followed by immunoprecipitation showed the presence of Rhop-H and SERA as seen in Fig 2 and reported previously in our other studies (Sam-Yellowe and Perkins 1990; Ndengele et al. 1995), where Rhop-H and SERA are precipitated by specific antibodies.

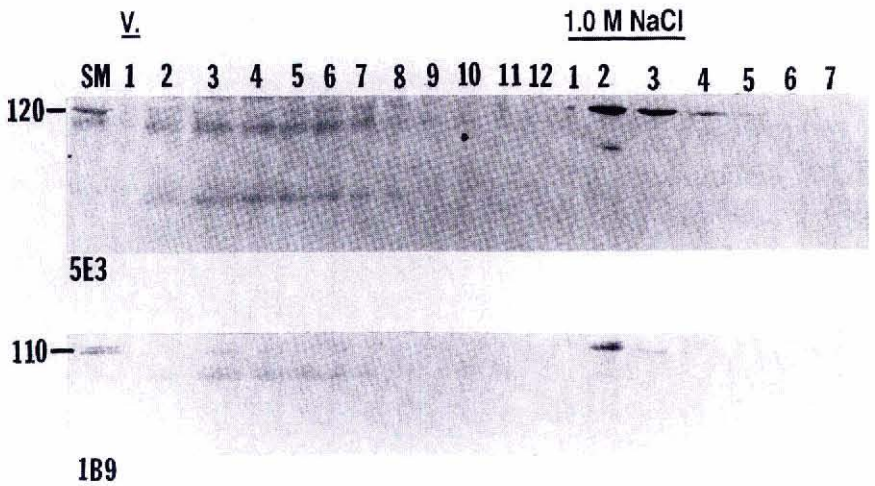

Figure 4. Affi-blue gel chromatography for enrichment of Rhop-H and SERA proteins. Spent culture supernatants collected from segmented schizonts were loaded on an affiblue column. Unbound proteins were collected in the void volume, the beads were washed and bound proteins were eluted with 1 M NaCl. Albumin was eluted with NaCNS. Twelve fractions were collected for each. All fractions were analyzed by immunoblotting with Mab 1B9, Mab 5E3 and antiserum K15. Rhop-3 and SERA were present in the SCS starting material (SM) and eluted by 1M NaCl in fractions 2-3. Both proteins were absent in the void (V) volume.

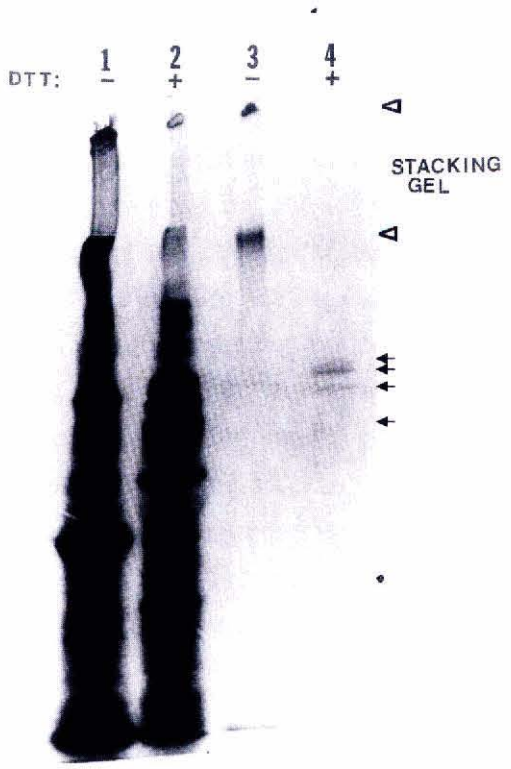

Figure 5. Chemical cross-linking analysis of Rhop-H and SERA proteins bound to mouse erythrocytes using DTSSP. Rhop-H and SERA proteins bound to mouse erythrocytes were cross-linked with DTSSP, eluted with 0.5 M NaCl and separated on a 5-15% SDS-PAGE gel under reduced and unreduced conditions. Lane 1, starting material culture supernatant (SCS) cross-linked and solubilized without DTT; lane 2, SCS cross-linked and solubilized with DTT; lane 3, NaCl eluate cross-linked and solubilized without DTT; lane 4, NaCl eluate solubilized with DTT. Rhop-H and SERA proteins bind in close proximity at a distance of 12 Å.

Rhop-H and SERA do not form a heteroligomeric complex in vivo

We performed cross-linking of parasite extracts followed by precipitation with specific antibodies and then separated the ICs by 2-D SDS-PAGE. These experiments were designed to investigate the spatial organization of native SERA and Rhop-H and to determine if both groups of proteins are associated. Sedimentation analysis of Rhop-H and SERA on sucrose gradients was also performed using schizont extracts. We wanted to know if both groups of proteins are associated in a complex in vivo before binding the erythrocyte surface, and secondly we wanted to know if both groups of proteins have different sedimentation rates in sucrose gradients. Cross-linked proteins analyzed by 2-D SDS-PAGE are shown in Figure 6. Following reduction in the second dimension, three proteins representing the individual Rhop-H proteins were identified with Rhop-H specific antibodies, Mab 1B9 (Fig. 6, panel 1), antiserum 686 (Fig. 6 panel 1, inset) (Sam-Yellowe et al. 1988; Yang et al.1996), and antiserum 680 (Sam-Yellowe et al. 1995), (results not shown). In the lanes with proteins separated in the first dimension, all three antibodies gave a similar pattern of reactivity (Fig. 6, Panel 1, lanes c-e), with precipitated proteins seen

in the same positions observed in the starting material either not cross-linked or cross-linked (panel 1, lanes a and b respectively). In lane d, following reduction of immune complexes containing cross-linked extracts, the 140/130/110 kDa proteins were seen resolved. However, immune complexes with cross-linked extracts solubilized without DTT showed very faint proteins resolved. Most of the cross-linked complex formed, remained at the top of the stacking gel (SG), lane e and did not enter the gel. Immune complexes separated in the second dimension show that the Rhop-H proteins were not associated with any other parasite proteins. Due to the absence of the IC in the first dimension, in samples containing cross-linked proteins, unreduced before electrophoresis, there were no proteins detected in the second dimension (panel 2, lane C). Control immunoprecipitates with Mab 1B9, using non cross-linked extracts, lane A and antiserum 686, lane C (inset) show similar bands of 140 kDa, 130 kDa and 110 kDa. Immune complexes with cross-linked extracts reduced with DTT (lane B, in large gel and inset) also showed Rhop-H proteins without SERA associated.

When SERA specific antibodies were used cross-linked products were identified in unreduced and reduced starting material samples (lanes a and b) and these resolved to a single protein of 120 kDa following reduction of ICs (lane d), suggesting that the 120 kDa SERA protein forms oligomeric complexes with other 120 kDa proteins (Fig. 6, panel 2). The 120 kDa protein in the cross-linked extract was identified with Mab 152.3F7.1.1, antiserum vP870 (Fig. 6, panel 2, large gel and inset, respectively), and antiserum 685 (results not shown). The Rhop-H proteins did not co-precipitate with SERA as seen in immunodepletion experiments. In control extracts before cross-linking and in cross-linked extracts immuoprecipitated by Mab 152.37.1.1 the 120 kDa protein was seen immunoprecipitated by specific antibody (panel 2, lanes c and d respectively). In lane e, ICs with cross-linked extracts unreduced with DTT did not enter the gel. Following reduction by DTT of cross-linked IC, the SERA protein was seen resolved in lane d. The partially resolved SERA protein in lane d shows that there were no other proteins associated with SERA. Similar results showing the SERA protein were obtained in the second dimension (panel 2, lane B, large gel and inset). However, a large amount of the cross-linked complex >300 kDa remained at the top of the well and did not enter the gel. For the same reasons indicated above, the SERA protein was not seen in the second dimension (Fig. 6, panel 2, lane A and C, inset and large gel, respectively).

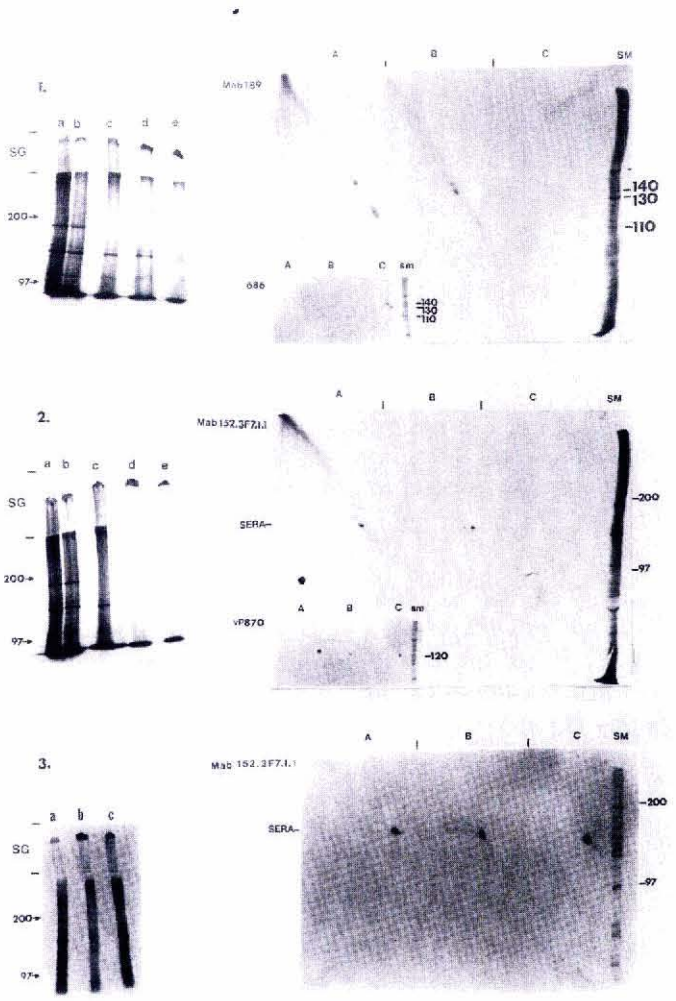

Figure 6. 2-D SDS-PAGE analysis of the spatial organization of Rhop-H and SERA in parasite extracts following chemical cross-linking with DTSSP. Schizont extracts were cross-linked with 5 mM DTSSP and immunoprecipitated with different antibodies specific for Rhop-H and SERA. Immune complexes of Rhop-H and SERA with specific antibodies were separated on 5% SDS-PAGE gel in the first dimension and lanes containing precipitated proteins were excised and separated in the second dimension on an 8% preparative SDS-PAGE gel. Panel 1, Control gel, lanes a, starting material (SM) extract before cross-linking; b, SM cross-linked with DTSSP; c, SM before cross-linking immunoprecipitated with Mab 1B9; d, Cross-linked SM immunoprecipitated with Mab 1B9 and reduced with DTT; e, Cross-linked SM immunoprecipitated with Mab 1B9 and separated without DTT reduction. SG=stacking gel. Large gel: immunoprecipitation with the Rhop-H specific antibodies #686 [21] and Mab 1B9 [8]. SM, starting material; A, control IC with 1B9 soluilized with DTT; B, IC with cross-linked extract immunoprecipitated with 1B9 and solubilized with DTT; C, IC with cross-linked extract immunoprecipitated with 1B9 and solubilized without DTT. Inset gel. A, IC with cross-linked extract immunoprecipitated with #686 and solubilized without DTT; B, IC with cross-linked extract immunoprecipitated with #686 and solubilized with DTT; C, control IC with #686 solubilized with DTT. Panel 2, Control

gel, lanes a, starting material (SM) extract before cross-linking; b, SM cross-linked with DTSSP; c, SM before cross-linking immunoprecipitated with Mab 152.3F7.1.1; d, Cross-linked SM immunoprecipitated with Mab 152.3F7.1.1 and reduced with DTT; e, Cross-linked SM immunoprecipitated with Mab 152.3F7.1.1 and separated without DTT reduction. SG=Stacking gel. Large gel: Immunoprecipitation with SERA specific antibodies Mab 152.3F7.1.1 and vP870 [19]. SM, starting material; A, control IC with Mab 152.3F7.1.1 solubilized with DTT; B, IC with cross-linked extract immunoprecipitated with Mab 152.3F7.1.1 and solubilized with DTT; C, IC with cross-linked extract immuno-precipitated with Mab 152.3F7.1.1 and solubilized without DTT. Inset gel. A, IC with cross-linked extract immunoprecipitated with vP870 and solubilized without DTT; B, IC with cross-linked extract immunoprecipitated with vP870 and solubilized with DTT; C, control IC with vP870 and solubilized with DTT. Panel 3 Control gel, lanes a, starting material (SM) extract before cross-linking; b, SM cross-linked with DTSSP and unreduced; c, SM cross-linked with DTSSP and reduced with DTT. SG=Stacking gel. A, IC with cross-linked extract immunoprecipitated with Mab 152.3F7.1.1 and solubilized without DTT; B, IC with cross-linked extract immunoprecipitated with Mab 152.3F7.1.1 and solubilized with DTT; C, control IC with Mab 152.3F7.1.1 and solubilized with DTT. Parasite proteins were labeled with high specific activity methionine and immunoprecipitated with high titer Mab 152.3F7.1.1. Gels were overexposed to detect any associated proteins. Asterisks are indicated near protein bands with low signals following fluorography.

Similar results were obtained in the second dimension (lane B, large gel and inset), where the SERA protein was seen unassociated with Rhop-H. Collectively, the data shows that SERA was complexed with itself in large oligomeric complexes. The use of schizont extracts labeled with higher specific activity of [^{35}S] methionine (Fig. 6, panel 3, lanes A, B and C) precipitated by higher titer Mab 152.357.1.1 and overexposed confirmed these results and showed very clearly that SERA is complexed with other SERA proteins and not Rhop-H by cross-linking. In this experiment, the amount of protein that entered the gel was detected by fluorography. These results indicate that spatially, Rhop-H and SERA proteins are not in close contact in vivo. It is possible that the presence of detergents used in the preparation of extracts for example, Triton X-100, may have also disrupted any associations between SERA and Rhop-H. Alternatively, amino acid groups required for interaction with reactive groups on the cross-linkers on both groups of proteins may not have been accessible, preventing association of the proteins by cross-linking. Furthermore, in the analysis of cross-linked proteins in structural studies, the type of cross-linker used and the conditions under which cross-linking was performed are crucial in obtaining interpretable data (Wood and O'Dorisio 1985; Wang and Richards 1974; Beckers et al. 1994; Peters and Richards 1977).

In the current studies we used different antibodies specific for each group of proteins to analyze cross-linked products and to overcome inconsistencies. Furthermore, experiments with each antibody were performed and analyzed by

immunoblotting and immunoprecipitation. Cross-linking has the advantage of demonstrating the structural relationship of proteins (Wood and O'Dorisio 1985; Wang and Richards 1974; Beckers et al. 1994; Peters and Richards 1977; Howard and Reese 1990). For the Rhop-H proteins, the IC appeared to be composed stoichiometrically of the individual Rhop-H proteins. However the data does not indicate in what form the proteins are associated. In previous studies, we showed that the binary complex of 110 /130 kDa proteins and the 130/140 kDa proteins may be in association while the 110 kDa and 140 kDa proteins may not be associated (Sam-Yellowe 1993). Since the Rhop-H complex is assembled immediately following synthesis of the individual proteins its association must exist in vivo. The SERA antigen is synthesized in the schizont stage but it does not associate with Rhop-H.

Rhop-H and SERA proteins have different sedimentation properties, and sedimentation does not require antibody interaction

The results in Fig. 7 show that prior interaction of native Rhop-H and SERA does not occur in extracts similar to the observation with SCS. Protein sedimentation occurred in the absence of specific antibodies. The Rhop-H proteins of 140/130/110 kDa sedimented at the denser region of the gradient (fractions 1-3) in the same fractions as myosin (200 kDa) and phosphorylase B (97.4 kDa), above carbonic anhydrase (29 kDa, 2.8 S) and bovine serum albumin (68 kDa, 4.6S). The complex of proteins sedimented together and was not distributed throughout the gradient. All three proteins were coprecipitated by Mab 1B9. Differential sedimentation of the individual Rhop-H proteins was not observed, suggesting that there was no dissociation of the proteins within the complex during ultracentrifugation. It appears that the stoichiometric composition of the Rhop-H complex is one molecule of Rhop-1, Rhop-2 and Rhop-3 associated in a complex as seen in our previous studies (Sam-Yellowe 1993). A similar sedimentation pattern was observed for the low molecular weight rhoptry protein complex, Rhop-L/RAP-1 (Howard and Reese 1990), suggesting that the protein complexes within the rhoptries possess similar characteristics. In the control IC, panel 2 lane a, Rhop-3/110 kDa appears to be more abundant. However, this did not affect the sedimentation rate of the complex and no dissociation of the complex was observed. We observed a high molecular weight protein band in the lighter densities of the gradient (panel 2, lanes e-j corresponding to fractions 4-8). However the protein was not seen associated with Rhop-H in ICs (panel 2, lanes b-d). At this time the identity of the protein is not known, and since the protein was observed in the lighter fractions, it seems unlikely that it is related to

Rhop-H. The SERA antigen was distributed over several gradient fractions from the dense to light regions of the gradient in fractions 4-9 (Fig. 7, panel 3, lanes e-j) but was not associated with Rhop-H proteins in ICs formed with antiserum vP870. We conclude from the sedimentation data that the Rhop-H complex and SERA have different sedimentation rates. The Rhop-H complex formation was not affected by extraction, disulfide bond reduction, centrifugation or by antibodies as observed previously (Sam-Yellowe 1993). It appears that SERA and Rhop-H function independently, and that prior association of SERA with Rhop-H proteins in vivo is not required for protein function. Based on the results of cross-linking on the surface of erythrocytes, we conclude that Rhop-H and SERA bind in close proximity to each other using different receptors and may probably function together during merozoite invasion, with each type of protein playing a specific role. Further investigations will be necessary to identify the specific receptors used by each protein.

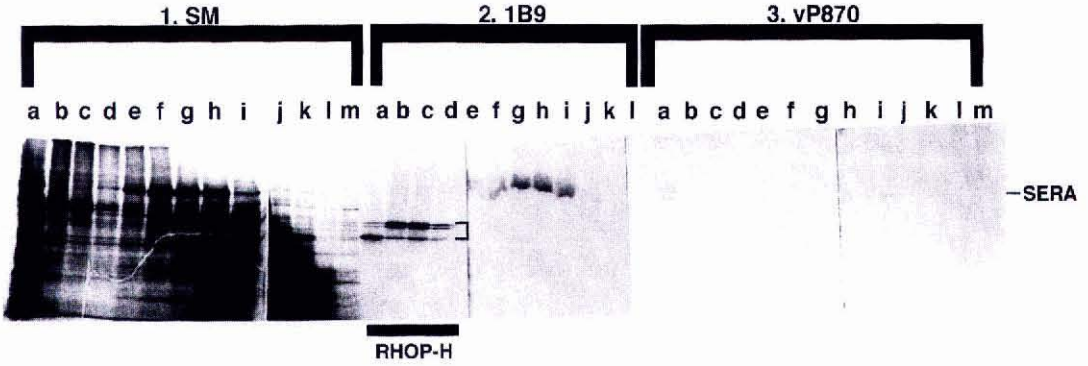

Figure 7. Sedimentation of Rhop-H and SERA proteins and analysis by immunoprecipitation with specific antibodies. Biosynthetically labeled schizonts extracted as described in TET buffer. Sonicated, centrifuged and loaded on a 5-20% continuous gradient. Fractions collected were immunoprecipitated with Mab 1B9 specific for Rhop-H proteins and rabbit antiserum vP870 specific for SERA. Panel 1, lane a, SM extract; lanes b-m, SM of fractions collected from gradient; Panel 2, lane SM immunoprecipitated by Mab 1B9; lanes b-l (fraction 12 was inadvertenlty cut and was similar to lane l), individual fractions immunoprecipitated by Mab 1B9; Panel C, lane a, SM immunoprecipitated by vP870; lanes a-m, individual fractions immunoprecipitaed by antiserum vP870.

In summary we have shown that both Rhop-H and SERA proteins are secreted into the SCS at the schizont stage and during merozoite release. Both group of proteins bind to specific independent receptors on the erythrocyte surface and are nearest neighbors on the cell surface, binding at a distance of approximately12 Å. In previous studies we and others showed that both groups of proteins also bind liposomes and associate with membranes (Ndengele et al. 1995; Perkins and Zeifer 1994; Sam-Yellowe and Perkins 1991; Ragge et al. 1990. Specific antibodies for each

type of protein showed no cross-reactivity or coprecipitation with heterologous proteins confirming that both Rhop-H and SERA do not interact to form complexes and that their interaction with the erythrocyte is by separate receptors. Taken together the results show that Rhop-H and SERA may play specific roles during parasite development, merozoite invasion and PVM formation. The erythrocyte binding domains on both groups of proteins may be important targets of immune attack in the infected host. These domains and the specific epitopes participating in binding and invasion are currently being investigated using recombinant proteins.

ACKNOWLEDGMENTS

Research support was by NIH grant AI3670 to TSY, Grant AI35827, the USAID (DPE-936-6001) and a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Sports and Culture of Japan to HF and MA. We thank William E. Brochak, Iris J. Cabrera and Celeste Perry for excellent technical assistance.

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