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Members of the Merozoite Surface Protein 7 Family with Similar Expression Patterns Differ in Ability To Protect against *Plasmodium yoelii* Malaria

Kerrianne Mello,[1]† Thomas M. Daly,[1] Carole A. Long,[2] James M. Burns,[1] and Lawrence W. Bergman[1]*

Division of Molecular Parasitology, Department of Microbiology & Immunology, Drexel University College of Medicine, Philadelphia, Pennsylvania,[1] and Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland[2]

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Previously, we described the isolation of the *Plasmodium yoelii* sequence-related molecules *P. yoelii* MSP-7 (merozoite surface protein 7) and *P. yoelii* MSRP-2 (MSP-7-related protein 2) by their ability to interact with the amino-terminal end of *P. yoelii* MSP-1 in a yeast two-hybrid system. One of these molecules was the homologue of *Plasmodium falciparum* MSP-7, which was biochemically isolated as part of the shed MSP-1 complex. In the present study, with antibodies directed against recombinant proteins, immunoprecipitation analyses of the rodent system demonstrated that both *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 could be isolated from parasite lysates and from parasite culture supernatants. Immunofluorescence studies colocalized *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 with the amino-terminal portion of MSP-1 and with each other on the surface of schizonts. Immunization with *P. yoelii* MSRP-2 but not *P. yoelii* MSP-7 protected mice against a lethal infection with *P. yoelii* strain 17XL. These results establish that both *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 are expressed on the surface of merozoites and released from the parasite and that *P. yoelii* MSRP-2 may be the target of a protective immune response.

Recently, many new protein molecules have been discovered on the surface of the malaria parasite, most belonging to the merozoite surface protein (MSP) family. Due to their surface exposure, they are accessible to antibodies and are therefore considered possible vaccine candidates (15, 23). Many of these surface proteins have been found to contain one or more epidermal growth factor-like domains, including MSPs 1, 4, 5, 8, and 10 (2, 3, 14, 28), some are soluble (MSP-3 and MSP-9) (24), and others have been identified as part of the shed MSP-1 complex (MSPs 6 and 7) (25, 27).

MSP-1 has been the most extensively characterized and examined for its potential biological function and possible role as a vaccine candidate. The protein is evenly distributed on the surface of the merozoite and undergoes a two-step proteolytic processing by a conserved membrane-associated protease (4, 5). MSP-1 is processed late in schizogony into 83-kDa, 30-kDa, 38-kDa, and 42-kDa fragments, which remain noncovalently associated on the surface of the parasite (14, 19, 21). The 42-kDa region at the carboxy terminus of the protein then undergoes a second proteolytic processing event into 33-kDa and 19-kDa fragments at the time of merozoite invasion. The 19-kDa region of the protein contains two epidermal growth factor-like domains and remains on the surface of the parasite through a glycosylphosphatidyl inositol anchor (6, 14). Immunization with the 19-kDa region of MSP-1 protects against lethal parasite challenge in mice and monkeys (8, 9, 10, 12, 16).

Recently, MSP-6 and MSP-7 have been found to be associated with the shed MSP-1 complex in *Plasmodium falciparum* (25, 27). MSP-7 is a protein with a predicted molecular mass of 22 kDa, expressed in late-stage parasites; the gene encoding this protein is on chromosome 13 and is part of a multigene family (22, 25). Previously, we used the yeast two-hybrid system to identify proteins that interact with the amino-terminal portion of *Plasmodium yoelii* MSP-1 and identified two sequence-related molecules, one of which is the homologue to MSP-7 originally described in *P. falciparum* (22, 25). Through BLAST analysis, we have identified six genes in *P. falciparum* that are the homologues to the *P. yoelii* genes isolated in the yeast two-hybrid screen and presented the molecular characterization of MSP-related proteins (MSRPs) 1, 2, and 3 in *P. falciparum*. In this study, we have undertaken the characterization of the *P. yoelii* homologues of MSP-7 and MSRP-2. We used the animal model to test the potential of these proteins to protect mice against lethal parasite challenge.

MATERIALS AND METHODS

Plasmid constructs. A 1,296-bp fragment corresponding to amino acids 82 to 514 of *P. yoelii* MSP-1_{83a} (18) was amplified from *P. yoelii* 17XL genomic DNA with primers containing *Eco*RI sites. The resulting fragment was cloned into the *Escherichia coli* expression vector pGEX4T-1 (Pharmacia Biotech), creating an in-frame fusion with glutathione *S*-transferase (GST). To express *P. yoelii* MSP-7 in *E. coli*, clone 6 from the library screen was digested with *Eco*RI and *Xho*I, and the resulting fragment of 828 bp (amino acids 47 to 324) was cloned in the poly(His) tag vector pET-28a (Novagen) for expression (22). Primers were designed to amplify a portion of library clone 5, corresponding to *P. yoelii* MSRP-2, lacking the proline- and serine-rich extension, resulting in a fragment of 789 bp

* Corresponding author. Mailing address: Department of Microbiology & Immunology, Drexel University College of Medicine, 2900 Queen Ln., Philadelphia, PA 19129. Phone: (215) 991-8376. Fax: (215) 848-2271. E-mail: Lawrence.Bergman@drexel.edu.

† Present address: Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115.

(amino acids 54 to 317). The resulting *Bam*HI-to-*Xho*I fragment was cloned into the pET-28a vector for expression. All sequences lack their amino-terminal signal peptides, and all DNA sequences and junctions were confirmed by DNA sequencing.

Expression and purification of recombinant proteins. The amino-terminal portion of *P. yoelii* MSP-1_{83a} was expressed as a fusion with GST, and *P. yoelii* MSP-7 and MSRP-2 were expressed as fusions with a six-histidine tag. All constructs were expressed in *E. coli* BL-21(DE3) Codon Plus cells (Stratagene). *P. yoelii* MSP-1_{83a} was purified under native conditions with glutathione agarose beads and eluted in 5.0 mM glutathione as previously described (22). *P. yoelii* MSP-1_{19} was expressed and purified as previously described as a fusion with GST (9, 10). *P. yoelii* MSP-7 and MSRP-2 were purified with nitrilotriacetic acid (NTA)-agarose (Qiagen) in a batch and column fashion according to the manufacturer's instructions. The purity and integrity of the proteins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie blue. Protein concentrations were determined by a Bradford assay (protein reagent; Bio-Rad).

Serum. Male BALB/cByJ mice 6 to 8 weeks old were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in our Association for Assessment and Accreditation of Laboratory Animal Care International-approved animal facility. For the production of polyclonal antisera, mice received three subcutaneous injections 3 weeks apart of 100 μg of recombinant protein (*P. yoelii* MSP-1_{83a}, *P. yoelii* MSP-7, or *P. yoelii* MSRP-2) with the Ribi adjuvant system (Corixa). Normal mouse serum was obtained from nonimmunized animals, and serum was obtained 2 weeks following the third immunization from the experimental groups.

Rabbit antisera against all three of the recombinant proteins was commercially prepared (Lampire Biological Laboratories, Pipersville, Pa.). The animals received three subcutaneous injections of 300 μg of recombinant protein with complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for the subsequent injections. The first and second immunizations were 3 weeks apart, and the second and third immunizations were 2 weeks apart. Serum was obtained 2 weeks following the final boost. Preimmune serum was obtained and screened prior to the immunizations.

Immunizations. Groups of four to eight male BALB/cByJ mice were immunized with 25 μg of the recombinant His fusions or 50 μg of the GST fusion proteins. In two challenge trials, the animals received three subcutaneous injections (of 25 μg or 50 μg each) of the recombinant protein with Ribi (Corixa) adjuvant 3 weeks apart. In the third challenge trial, the animals received three subcutaneous injections of recombinant protein with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the two boosting immunizations 3 weeks apart. All groups were challenged intravenously 2 weeks following the last boost with 10^4 parasitized erythrocytes. Control animals were immunized with their respective adjuvant in phosphate-buffered saline (PBS).

Parasites and experimental infections. *P. yoelii* 17XL was maintained as a cryopreserved stabilate. Blood stage infections were initiated by intraperitoneal injection of parasitized erythrocytes into a donor animal, and infections were monitored on a daily basis by thin tail blood smears and Giemsa staining between days 5 and 25 postinfection. An average of 300 cells were counted per slide. Animals were removed from the study when their parasitemia exceeded 50% or they were obviously moribund.

Indirect immunofluorescence. Parasitized blood was collected from mice infected with *P. yoelii* 17XL when the parasitemia was 20 to 25%. Parasitized cells were washed once and separated on a Percoll gradient to collect the late stages. Cells were washed once in PBS, pelleted, and resuspended in equal volumes of PBS. Thin blood smears were prepared, air dried, and fixed in methanol-acetone (1:1) for 20 min at −20°C. Slides were air dried and stored at −20°C until used. Slides were hydrated for 5 min at room temperature in PBS. Fixed cells were incubated for 30 min at 37°C in a humidified chamber with normal mouse serum, immune mouse serum, preimmune rabbit serum, or immune rabbit serum at a 1:100 dilution in PBS. Slides were washed three times in PBS for 5 min with agitation. The slides were then incubated as described above with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma) with 10% normal goat serum (Gibco) diluted 1:100 in PBS and rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:100 in PBS. Combination slides with *P. yoelii* MSRP-2 and *P. yoelii* MSP-1_{83a} or *P. yoelii* MSP-7 and *P. yoelii* MSP-1_{83a} were incubated separately with their primary and secondary antibodies, while the combination slides with *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 were incubated simultaneously with their primary antiserum and separately with their secondary antibodies. Slides were washed three times with PBS and incubated for 15 min at 37°C with Hoechst cell stain at a 1:1,000 dilution. Slides were washed three times for 5 min with PBS, mounted with an antifade solution (Molecular Probes), sealed, and visualized.

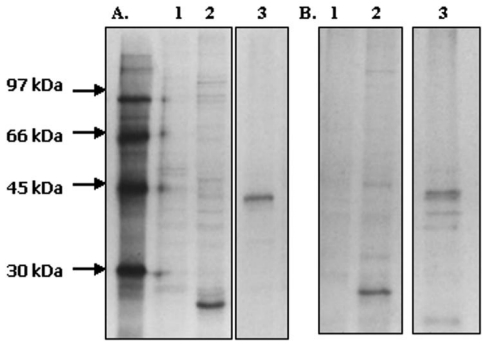

FIG. 1. Immunoprecipitation with late-stage *P. yoelii* 17XL parasites and culture supernatants labeled in vitro for 6 h. (A) Immunoprecipitations with [^{35}S]methionine-cysteine-labeled *P. yoelii* 17XL antigen. Lane 1, normal mouse serum; lane 2, mouse anti-*P. yoelii* MSP-7; lane 3, mouse anti-*P. yoelii* MSRP-2. (B) Immunoprecipitations with ^{35}S-labeled culture supernatant. Lane 1, normal mouse serum; lane 2, mouse anti-*P. yoelii* MSP-7; lane 3, mouse anti-*P. yoelii* MSRP-2. All samples were preabsorbed with preimmune serum and exposed to film for 4 weeks.

Antibody assay. Enzyme-linked immunosorbent assays (ELISAs) were performed to measure prechallenge antibody responses. Serum samples were collected from the tail vein 2 to 3 days prior to parasite challenge; each well of a 96-well flat-bottomed plate was coated with 50.0 ng of recombinant protein in 0.1 M sodium carbonate, pH 9.6, overnight at 4°C. The plates were washed three times with PBS and 0.1% Tween 20. Prechallenge serum was serially diluted and assayed. The serum was incubated for 30 min at room temperature and then washed three times with 1× PBS with 0.1% Tween 20. A goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody was incubated for 30 min at room temperature at a dilution of 1:2,000. The plate was then washed four times with PBS with 0.1% Tween 20. The plate was developed at room temperature with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Promega). Color development was stopped with 1 M HCl and read at an optical density of 450 nm. ELISA reactions from adjuvant-only controls were performed and subtracted as background from each assay.

Radiolabeling and immunoprecipitations. Labeling of *P. yoelii*-parasitized erythrocytes was done in a 1-ml volume in a 24-well plate; 200 μl of parasitized erythrocytes was added to 800 μl of modified minimal essential medium without methionine and cysteine, 50.0 mM HEPES (pH 7.5), and 100 to 200 μCi of [^{35}S]methionine-cysteine (Perkin Elmer). The plate was gassed with 5% CO_2 and incubated at 37°C for 6 h. Labeled material was centrifuged at 500 × *g* for 10 min to pellet the cells. Culture supernatant and pellets were frozen at −80°C. The pellets were thawed and resuspended in 5 ml of ice-cold lysis buffer (20.0 mM Tris-HCl [pH 7.5], 50.0 mM NaCl, 5.0 mM EGTA, 1% Brij 58, 0.5% deoxycholate, and 1.0 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 to 20 min. Solutions were then pelleted in the ultracentrifuge at 25,000 × *g* for 1 h; 25 μl of the soluble radiolabeled material and culture supernatant were used in trichloroacetic acid precipitations.

From 1×10^5 to 3×10^5 cpm of radiolabeled soluble antigen or culture supernatant was used per immunoprecipitation reaction. *P. yoelii* immunoprecipitations used 200 μl of soluble antigen and 150 μl of radiolabeled supernatant in their respective reactions. All samples were preabsorbed with normal mouse serum and then incubated with 1 to 5 μl of their respective antiserum for 30 min on ice. Samples were then incubated with 20 μl of protein A-Sepharose (Sigma) as the absorbent for 30 min on ice with mixing. Samples were than underlaid with 200 μl of immunoprecipitation buffer (20.0 mM Tris-HCl [pH 7.5], 50.0 mM NaCl, 5.0 mM EDTA, 0.5% Triton X-100, 0.5% deoxycholate, and 1.0 mM phenylmethylsulfonyl fluoride) containing 1.0 M sucrose. The sample was then centrifuged for 3 min at 5,000 × *g*. Beads were washed three times with 200 μl of immunoprecipitation buffer and finally resuspended in 20 μl of 2× SDS loading buffer. Samples were boiled for 5 min and separated on an SDS–10% PAGE gel. The proteins were fixed for 30 min by incubating the gel with 10% acetic acid and 50% methanol at room temperature with agitation. The gel was then incubated in Amplify solution (Amersham NAMP 100) for 30 min at room temperature with agitation. Finally, the gel was dried for 1 h at 80°C and exposed to film at −80°C for 1.5 to 4 weeks.

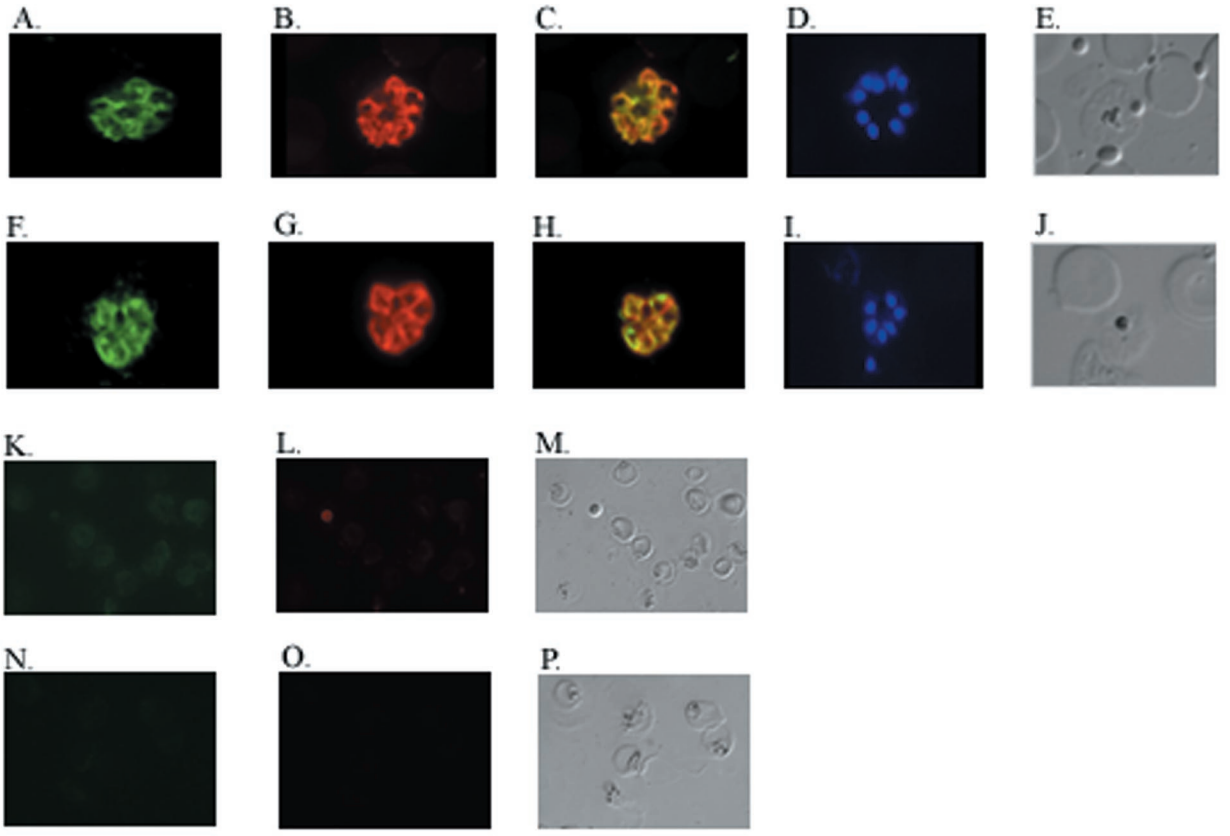

FIG. 2. Colocalization of *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 with *P. yoelii* MSP-1_{83a}. Shown are immunofluorescence assay results for thin blood smears of *P. yoelii* 17XL parasites. The slides were incubated with reagents. (A) Rabbit anti-*P. yoelii* MSRP-2 and a fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody. (B) Mouse anti-*P. yoelii* MSP-1_{83a} and a rhodamine-labeled goat anti-mouse IgG secondary antibody. (C) Overlay of panels A and B. (D) Hoechst cell staining. (E) Bright field of panels A to D. (F) Rabbit anti-*P. yoelii* MSP-7 and a fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody. (G) Mouse anti-*P. yoelii* MSP-1_{83a} and a rhodamine-labeled goat anti-mouse IgG secondary antibody. (H) Overlay of panels F and G. (I) Hoechst cell staining. (J) Bright field of panels F to I. (K) Preimmune rabbit antisera and a fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody. (L) Normal mouse antiserum and rhodamine-labeled goat anti-mouse IgG secondary antibody. (M) Bright field of panels K and L. (N) Preimmune rabbit antiserum and fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody. (O) Normal mouse antiserum and rhodamine-labeled goat anti-mouse IgG secondary antibody. (P) Bright field of panels N and O.

Statistical analysis. The Fisher exact probability test was used to determine the statistical significance in the difference in the numbers of surviving animals between the immunized and control groups. A Mann-Whitney U test determined the statistical differences between the peak parasitemias and between prechallenge antibody responses. GraphPad Instat (GraphPad Software, Inc.) was used to perform the statistical analysis.

RESULTS

Immunoprecipitations. The amino-terminal portions of *P. yoelii* MSP-1_{83a}, *P. yoelii* MSRP-2, and *P. yoelii* MSP-7 were expressed as fusion proteins in *E. coli*. *P. yoelii* MSP-1_{83a} was expressed as a GST fusion, and *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 were expressed as His fusions. The purified proteins were used to generate specific antisera in mice and rabbits. The ability of our polyclonal antisera to detect *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 in parasite samples and culture supernatant was assessed by immunoprecipitation reactions. Holder and colleagues found that MSP-7 and MSP-6 in *P. falciparum* were associated with the shed MSP-1 complex (25, 27). Late-stage *P. yoelii* 17XL parasites were labeled in vitro with [^{35}S]methionine-cysteine. Labeled culture supernatant and solubilized parasites were then used in immunoprecipitation reactions with polyclonal mouse serum against recombinant *P. yoelii* MSRP-2 and *P. yoelii* MSP-7.

The antiserum was able to precipitate *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 from both the radiolabeled culture supernatant and the soluble antigen (Fig. 1). The apparent molecular mass (approximately 26 kDa) of *P. yoelii* MSP-7 is slightly smaller than that of the recombinant protein, suggesting that the protein has undergone processing. Furthermore, from these studies it appears that the antisera are specific for each protein and do not cross-react. This has been confirmed by Western analysis with the purified recombinant proteins (data not shown). Normal mouse serum showed minimal reactivity with samples. These results indicate that *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 are shed into the culture supernatant, similar to the proteolytic fragments of *P. falciparum* MSP-1, and that the proteins can be detected in solubilized parasite material.

Localization of *P. yoelii* MSRP-2 and *P. yoelii* MSP-7. The localization of *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 was assessed with indirect immunofluorescence. *P. yoelii* 17XL blood

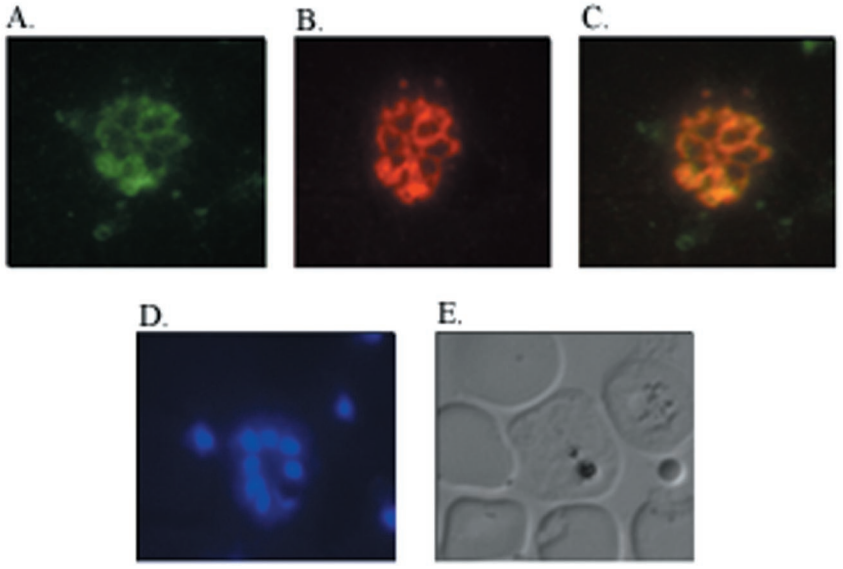

FIG. 3. Colocalization of *P. yoelii* MSP-7 and *P. yoelii* MSRP-2. Shown is immunofluorescence on thin blood smears of *P. yoelii* 17XL parasites. The slides were incubated with rabbit anti-*P. yoelii* MSP-7 followed by a fluorescein isothiocyanate-labeled goat anti-rabbit IgG secondary antibody or (A) mouse anti-*P. yoelii* MSRP-2 followed by a rhodamine-labeled goat anti-mouse IgG secondary antibody (B). (C) Overlay of panels A and B. (D) Hoechst cell staining. (E) Bright field of panels A to D.

stage parasites were enriched for late stages by Percoll separation and used to make thin smears that were fixed and air dried. The cell stage was assessed by counting nuclei with Hoechst cell staining (Fig. 2D and I). *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 were localized to the surface of schizonts, and this pattern is similar to that observed with the amino-terminal portion of *P. yoelii* MSP-1 (Fig. 2A, B, F, and G). Figure 2C shows an overlay and colocalization of *P. yoelii* MSRP-2 with *P. yoelii* MSP-1_{83a}, and Fig. 2H shows an overlay and colocalization of *P. yoelii* MSP-7 and *P. yoelii* MSP-1_{83a}. No fluorescent signal was detected in the normal mouse serum or the preimmune rabbit serum (Fig. 2K to P). Interestingly, combined indirect immunofluorescence also revealed that *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 are expressed on the same late-stage parasitized cell at the same time (Fig. 3). These results demonstrate that *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 are coexpressed on the surfaces of late blood stage parasites and they colocalize with the amino-terminal portion of MSP-1.

Immunization and challenge experiments. In an effort to assess the potential of *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 to protect mice against lethal parasite challenges, three challenge experiments were undertaken, immunizing groups of mice with *P. yoelii* MSRP-2 or *P. yoelii* MSP-7. In the first two trials, the proteins were administered in combination with Ribi as an adjuvant. Parasitemia was monitored on a daily basis, and the animals were removed from the study when their parasitemia exceeded 50% or they were obviously moribund. In trial 1, the mice were injected with 25.0 μg of recombinant *P. yoelii* MSRP-2 or *P. yoelii* MSP-7. Control animals were injected with Ribi in PBS, and all mice in the control group were removed from the study by day 8 postchallenge due to parasite burden. The *P. yoelii* MSP-7 experimental group was also removed from the study at day 8 postchallenge (Fig. 4A and B), whereas four out of five animals immunized with *P. yoelii* MSRP-2 survived challenge (Fig. 4C).

The *P. yoelii* MSRP-2 group exhibited two waves of parasitemia. Mean peak parasitemia data were analyzed on day 8 postinfection before the removal of both the control group and the *P. yoelii* MSP-7 experimental group. The differences in mean peak parasitemias between the groups did not reach statistical significance, but the number of surviving animals in the *P. yoelii* MSRP-2 group versus those of the control group and the *P. yoelii* MSP-7 group was statistically significant, with a *P* value of 0.0476 as determined by a Fisher exact probability test.

In trial 2, mice were immunized as in trial 1 with the addition of a group immunized with 60.0 μg of *P. yoelii* MSP-1_{19}. The number of surviving animals was similar to the results of trial 1. All five control animals and *P. yoelii* MSP-7 animals succumbed to infection (Fig. 5A and B). Three out of the five animals immunized with *P. yoelii* MSP-1_{19} survived infection, with one animal displaying a parasitemia below 1% and then clearing the infection over the duration of the experiment (Fig. 5C), while four out of five animals immunized with *P. yoelii* MSRP-2 survived challenge and again displayed the two waves of parasitemia seen in the first trial (Fig. 5D).

In the second trial with Ribi, the difference in mean peak parasitemia was examined on day 9 postinfection, but it was not quite statistically significant between the animals immunized with *P. yoelii* MSRP-2 and the control animals, with a *P* value of 0.0556 (19.44% versus 46.66%). There was a statistically significant difference between the mean peak parasitemia of the *P. yoelii* MSRP-2 group and that of the *P. yoelii* MSP-7 experimental group, with a *P* value of 0.0317 (19.44% versus 51.28%). The survival rate of the *P. yoelii* MSRP-2 group was statistically significant compared to those of the control group and the *P. yoelii* MSP-7 group, with a *P* value of 0.0476. Overall, in the two trials with Ribi adjuvant, *P. yoelii* MSRP-2 protected better against lethal challenge with *P. yoelii* 17XL, with an 80% survival rate, compared to *P. yoelii* MSP-7, with a 0% survival rate (see Fig. 7A and B).

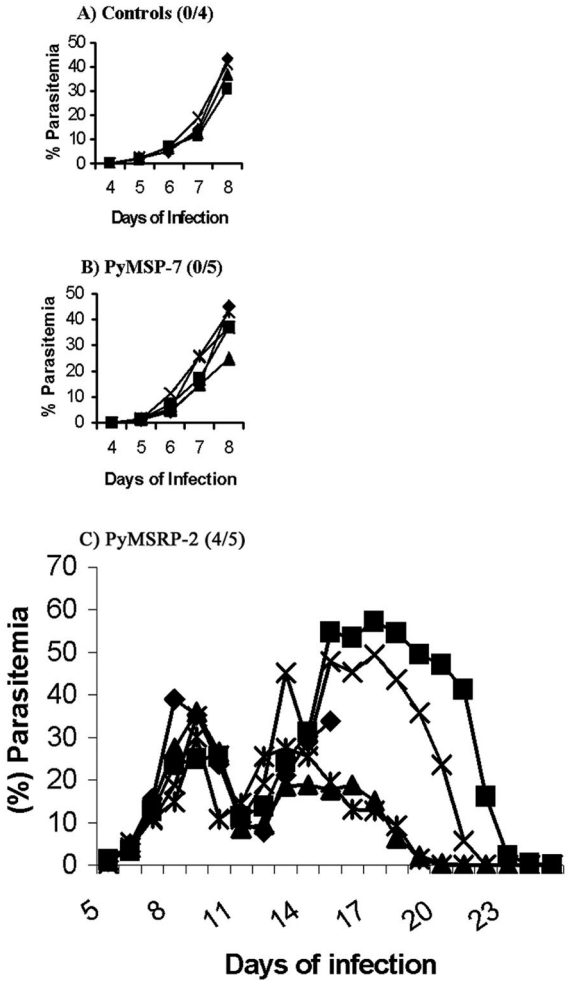

FIG. 4. Blood stage parasitemia in groups of mice for trial 1 with Ribi adjuvant. The number of survivors out of the total number of mice per group is shown in parentheses next to the name of the protein administered.

In attempts to improve protection, the animals received the same doses of recombinant proteins in combination with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the boosting immunizations. The number of animals in the *P. yoelii* MSP-7 and *P. yoelii* MSRP-2 experimental groups was increased from five to eight. All four control mice succumbed to infection (Fig. 6A). Different regions of *P. yoelii* MSP-1 were also used to immunize animals to assess protective efficacy, and four out of five animals immunized with the 19-kDa region of *P. yoelii* MSP-1 survived challenge, while zero out of five mice immunized with the amino-terminal portion of the 83-kDa region of *P. yoelii* MSP-1 survived parasite challenge (Fig. 6B and C).

There was a statistically significant difference in the mean peak parasitemias between the *P. yoelii* MSP-1_{19} and the control animals on day 8 postinfection, with a *P* value of 0.0159 (0.58% versus 38.47%). In the group immunized with *P. yoelii* MSRP-2, six out of the eight animals survived challenge while only two out of the eight animals immunized with *P. yoelii* MSP-7 survived challenge (Fig. 6D and E). The difference in mean peak parasitemia on day 8 postinfection reached statistical significance when the *P. yoelii* MSRP-2 group was compared to the control animals, with a *P* value of 0.0242 (11.08% versus 38.47%), but when the mean peak parasitemia of the *P. yoelii* MSRP-2 group was compared to that of the group immunized with *P. yoelii* MSP-7 on day 8 postinfection, the *P* value was 0.232 (11.08% versus 19.02%). There was a statistical difference in mean peak parasitemia when the *P. yoelii* MSRP-2-immunized group was compared with the *P. yoelii* MSP-1_{83a}-immunized group, with a *P* value of 0.01 (11.08% versus 37.94%). Five out of the eight mice in the *P. yoelii* MSRP-2 group exhibited parasitemia below 10%, and none of the animals exhibited the second wave of parasitemia. *P. yoelii* MSRP-2 gave a 75% survival rate when administered with Freund's adjuvant. This is significantly better than the 25% survival rate observed when the animals were immunized with *P. yoelii* MSP-7, indicating that *P. yoelii* MSRP-2 is better at protecting animals from lethal challenge with *P. yoelii* 17XL (Fig. 7C).

ELISA analysis on prechallenge antibody responses. Prechallenge antibody responses were analyzed by ELISA of the antigen used in the immunizations. Small amounts of serum were collected from the tail vein 2 to 3 days prior to infection with parasites. The antigens were coated on the bottom of a 96-well flat-bottomed plate overnight at 4°C, and the serum was tested at a 1:200 dilution in replicate. Assays were developed with a horseradish peroxidase substrate and read at an absorbance of 450 nm. In trial 1 with Ribi, there was no difference in the prechallenge responses between the experimental groups. On average, the *P. yoelii* MSRP-2 group had an optical density at 450 nm (OD_{450}) of 0.182, and the *P. yoelii* MSP-7 group had an average OD_{450} reading of 0.137 (Fig. 8A). In the second trial, the prechallenge antibody responses were slightly increased, with average readings of 0.33 for *P. yoelii* MSRP-2 and 0.35 for *P. yoelii* MSP-7, and the *P. yoelii* MSP-1_{19} experimental group had an average prechallenge antibody response OD_{450} reading of 0.683 (Fig. 8B). These results indicate that the prechallenge antibody levels between the *P. yoelii* MSP-7 and the *P. yoelii* MSRP-2 groups were not significantly different.

In the third trial with complete Freund's adjuvant, the prechallenge antibody responses were quite similar, with the *P. yoelii* MSRP-2 group having an average reading of 0.697 and the *P. yoelii* MSP-7 group having an average reading of 0.72. The prechallenge antibody responses in the *P. yoelii* MSP-1_{19} and MSP-1_{83a} groups had average readings of 0.494 and 0.442, respectively (Fig. 8C). Overall, the prechallenge antibody response in the complete Freund's adjuvant trial was slightly higher in the *P. yoelii* MSRP-2 and *P. yoelii* MSP-7 individual groups than that previously seen with the Ribi adjuvant. However, there were no statistically significant differences between the prechallenge antibody levels in the *P. yoelii* MSRP-2 group and those in the *P. yoelii* MSP-7 immunized group or between the groups of animals immunized with *P. yoelii* MSP-1_{19} and *P. yoelii* MSP-1_{83a}.

DISCUSSION

Through a combination of the genome project and molecular approaches, there has been continuous discovery and characterization of proteins associated with the malaria parasite. Previously, we used the yeast two-hybrid system to identify

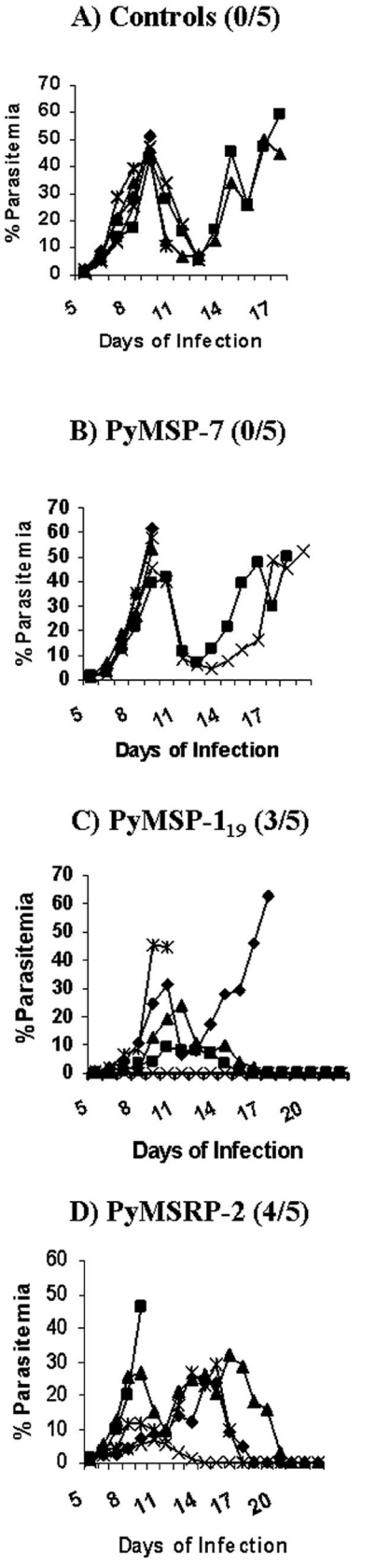

FIG. 5. Blood stage parasitemia in groups of mice for trial 2 with Ribi adjuvant. The number of survivors out of the total number of mice per group is shown in parentheses next to the name of the protein administered.

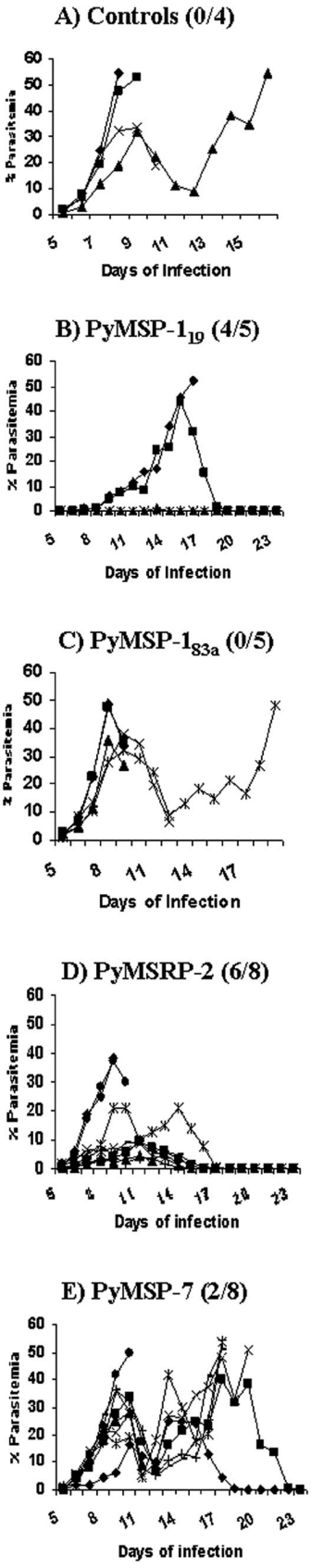

FIG. 6. Blood stage parasitemia in groups of mice for trial 3 with complete Freund's adjuvant. The number of survivors out of the total number of mice per group is shown in parentheses next to the name of the protein administered.

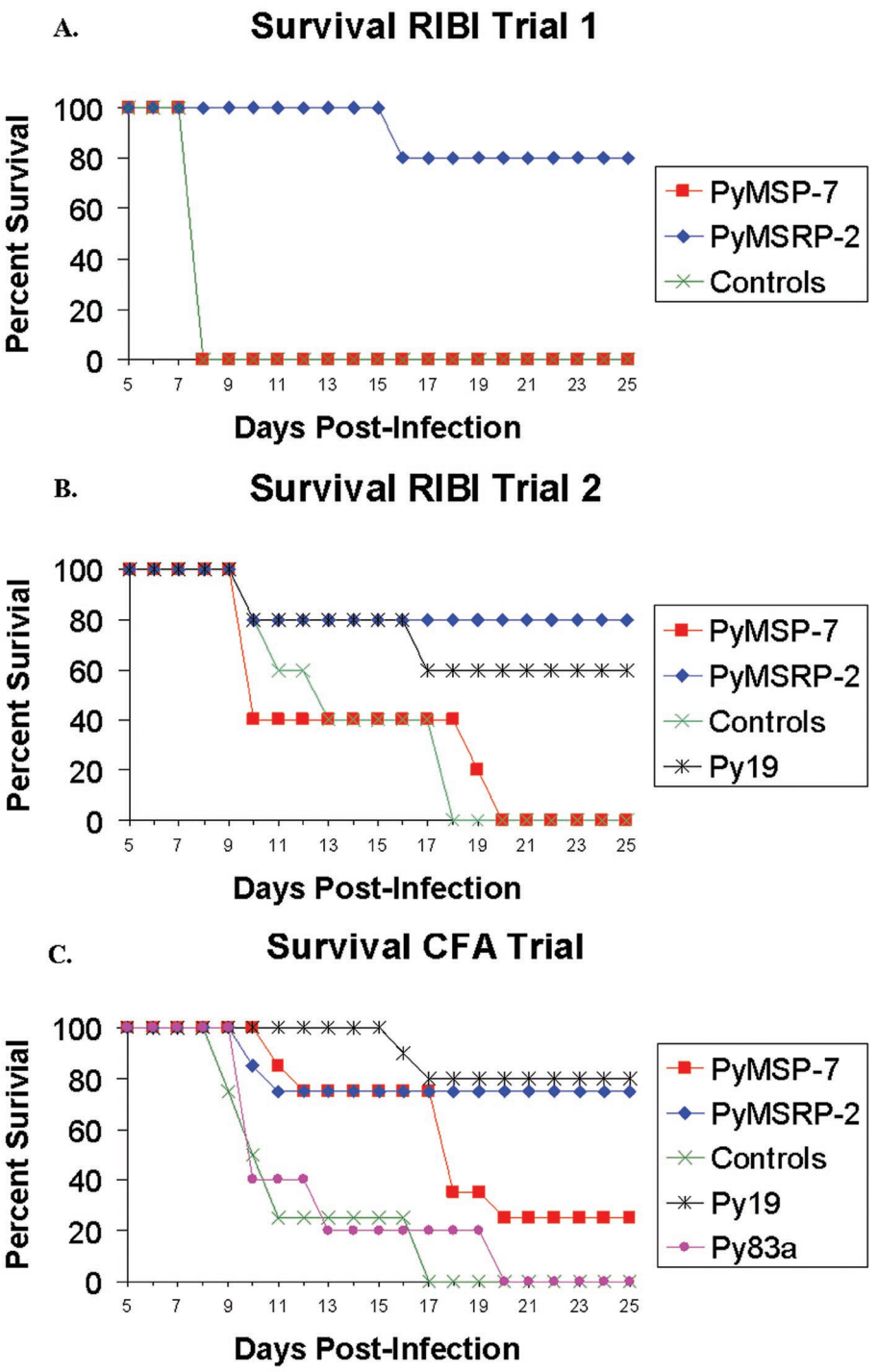

FIG. 7. Survival curves for challenge experiments. (A) Survival curves for animals in the first trial with Ribi. (B) Trial 2 with Ribi. (C) Trial with complete Freund's adjuvant.

proteins that associate with a portion of the amino-terminal 83-kDa proteolytic fragment of *P. yoelii* MSP-1 and characterized several of the homologues present in *P. falciparum* (22). In this study, we focused our attention on the characterization of the *P. yoelii* genes MSP-7 (homologue to MSP-7 originally described in *P. falciparum*) and MSRP-2.

Analysis of the genes revealed that they are 25% identical and 43% similar and lie adjacent to one another on the same contig (22). Immunoprecipitation studies demonstrated that *P. yoelii* MSRP-2 could be precipitated from radiolabeled parasite material and culture supernatants. *P. yoelii* MSRP-2 appears as a band of approximately 45 kDa, which corresponds to the molecular mass of the full-length protein. This was not the case when immunoprecipitation assays were performed with

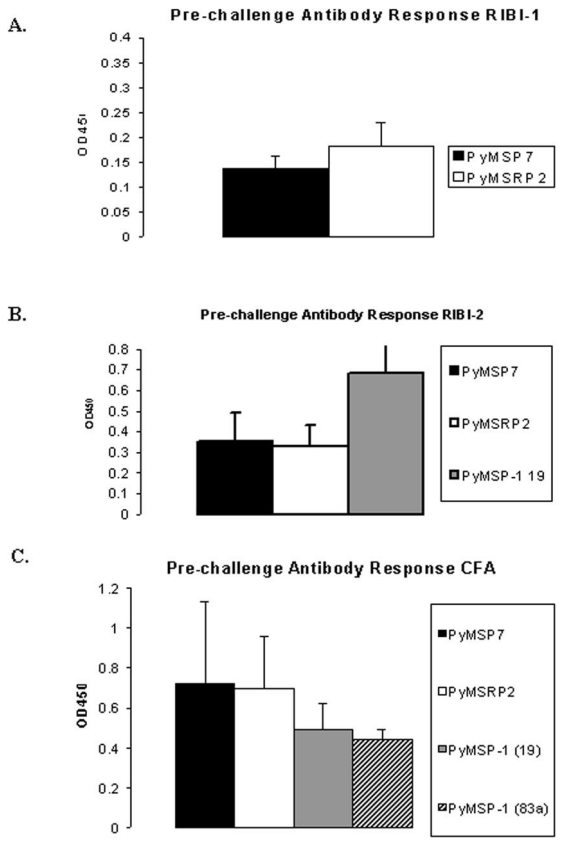

FIG. 8. Prechallenge antibody responses of mice in trials 1, 2, and 3 (A, B, and C, respectively). Values are indicated as optical density at 450 nm. Each bar represents the average reading for the experimental group at a 1:200 dilution.

antisera specific for *P. yoelii* MSP-7. The protein was precipitated as a band of approximately 22 kDa, which is smaller than the predicted molecular mass of the full-length protein at 35.5 kDa, suggesting that it has undergone processing, a finding consistent with the processing that occurs with MSP-7 in *P. falciparum* (25).

It is unclear why the immunoprecipitation reactions with antisera directed against either MSRP-2 or MSP-7 fail to coprecipitate additional components of the MSP-1 complex (i.e., MSP-1 itself). The association between the proteins may be stabilized in some way while on the parasite surface and weaken once the molecules are shed into the culture supernatant, or they may be disrupted when the parasites are solubilized by the use of detergents in the immunoprecipitation buffers. With specific antisera directed against *P. yoelii* MSP-7 and *P. yoelii* MSRP-2, we have shown that these molecules are expressed on the surface of late-stage intraerythrocytic parasites colocalizing with MSP-1 and each other.

Protective immune responses are generated in mice immunized with *P. yoelii* MSRP-2 but not *P. yoelii* MSP-7. When the challenge trials are compared, the group immunized with *P. yoelii* MSRP-2 and complete Freund's adjuvant have statistically significant decreases in parasitemia and do not display the second wave of infection that was observed when the protein was administered in combination with Ribi as the adjuvant. In groups immunized with *P. yoelii* MSRP-2, there is not 100% survival of the animals, and the Ribi group exhibits higher parasitemia than the complete Freund's adjuvant groups. This presents one of the obstacles in malaria vaccine development. Complete Freund's adjuvant is the only adjuvant that has reliably induced protective immunity in monkeys, but it is not suitable for human use (13). Other groups have also demonstrated that the successful protection of mice in challenge experiments was dependent on the adjuvant, and in some cases protection was dependent on the genotype of the animal (11).

One potential reason for the increased protective immune responses resulting from immunization with *P. yoelii* MSRP-2 compared to *P. yoelii* MSP-7 is that significantly less *P. yoelii* MSP-7 is present on the surface of the parasite. However, real-time PCR analysis has suggested that the mRNA for *P. yoelii* MSP-7 is approximately twofold more abundant than the mRNA for *P. yoelii* MSRP-2 and that *P. yoelii* MSP-1 mRNA is approximately 3.7-fold more abundant than the mRNA for *P. yoelii* MSP-7 and 6.5- to 6.9-fold more abundant than the mRNA for *P. yoelii* MSRP-2 (data not shown).

One way to increase protection may be to consider a multicomponent vaccine composed of several antigens from the same or different stages of parasite development. Recently, administering mice a combination of *P. yoelii* MSPs 4 and 5 with the 19-kDa region of *P. yoelii* MSP-1 provided enhanced protection in mice compared to immunizing with *P. yoelii* MSPs 4 and 5 or *P. yoelii* MSP-1_{19} alone (17). In preliminary studies, *P. yoelii* MSRP-2 was used to immunize animals in combination with the amino-terminal portion of *P. yoelii* MSP-1_{83a} or *P. yoelii* MSP-7, and the animals fared worse (data not shown). Prechallenge antibody levels do not seem to play a role in protection, as there was no significant difference between the levels of antibody in the protected and the unprotected groups of animals. Other possibilities for the observed protection may rely upon the isotype or avidity of the antibodies produced by immunization with the different proteins.

The precise role of the MSRP family members is unclear. The challenge data suggest that at least *P. yoelii* MSRP-2 may have a role in the invasion process. By blocking *P. yoelii* MSRP-2, one could be blocking the function of MSP-1 though its association with *P. yoelii* MSRP-2 on the surface of the parasite and inhibiting the ability of the parasite to invade cells. Alternatively, antibodies to the surface proteins might be causing the agglutination of the merozoites, thereby inhibiting their invasion. The mechanism(s) by which immunization with *P. yoelii* MSRP-2 provides protection needs to be examined further. The contribution of the other parts of the immune system, such as cell-mediated immunity, cannot be discounted. Examining the role or function of these various surface proteins will lead to a better understanding of the biology of the parasite and open new avenues for vaccine development.

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