

Multiple *Plasmodium falciparum* Merozoite Surface Protein 1 Complexes Mediate Merozoite Binding to Human Erythrocytes*

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Successful invasion of human erythrocytes by *Plasmodium falciparum* merozoites is required for infection of the host and parasite survival. The early stages of invasion are mediated via merozoite surface proteins that interact with human erythrocytes. The nature of these interactions are currently not well understood, but it is known that merozoite surface protein 1 (MSP1) is critical for successful erythrocyte invasion. Here we show that the peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 bind directly to MSP1, but independently of each other, to form multiple forms of the MSP1 complex on the parasite surface. These complexes have overlapping functions that interact directly with human erythrocytes. We also show that targeting the p83 fragment of MSP1 using inhibitory antibodies inhibits all forms of MSP1 complexes and disrupts parasite growth *in vitro*.

Invasion of merozoites into human erythrocytes is mediated through a series of interactions from distinct classes of proteins. During the early stages of invasion, merozoites come into contact with human erythrocytes, where merozoite surface proteins (MSPs)² are thought to be the primary ligands responsible for these low-affinity interactions (reviewed in Ref. 1). The most abundant MSP is the glycosylphosphatidylinositol (GPI)-anchored merozoite surface protein 1 (MSP1) (2). It is a 190- to 200-kDa protein that is proteolytically cleaved into four fragments (p42, p38, p30, and p83) by PfSub1, a subtilisin-like protease (3, 4). The four fragments are held together through non-covalent interactions on the surface of the parasite, anchored through a GPI anchor on the p42 fragment. Fragments of MSP1 have been implicated in binding directly to both band 3 and glycophorin A on the red blood cell surface (5, 6). During invasion, a second subtilisin-like protease, PfSub2, cleaves the MSP1 protein on the C-terminal end of the p42 fragment, leaving a 19-kDa stub (p19) that is retained on the parasite surface after entry into the erythrocyte (7). The remaining ectodomain

of MSP1 is shed into the bloodstream following invasion (8–12). Other peripheral MSPs, such as MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7, interact directly with MSP1 on the surface of parasites and are also shed following invasion (13–17).

It has previously been proposed that several peripheral MSPs, including MSPDBL1, MSPDBL2, and MSP3, act as ligands for erythrocyte receptors. The peripheral proteins MSPDBL1 and MSPDBL2 bind to MSP1 on the parasite surface and facilitate binding to red blood cells (17–19). In a study utilizing peptide fragments for MSP3, synthetic MSP3 peptides were shown to have some involvement in binding to host erythrocytes (20). However, the role of other peripheral surface proteins that are known to bind to MSP1, such as MSP6 and MSP7, remains poorly understood. It is unclear whether all of these peripheral MSPs contribute directly to the invasion process, although the ability to disrupt the corresponding gene encoding these peripheral MSPs without any detrimental effect on parasite growth is consistent with them having overlapping functions. It is also unclear whether all of the peripheral proteins are located in one large macromolecular complex or whether multiple forms of the MSP1 complex exists on the parasite surface.

Merozoite surface proteins present themselves as promising vaccine candidates because of their location on the parasite surface and exposure to the host immune system. Antibodies against MSPs function *in vivo* to decrease parasitemia either by directly blocking parasite invasion, opsonizing merozoites for phagocytosis, or inducing antibody-dependent cellular inhibition. In growth inhibition assays, antibodies against all processing fragments of MSP1 as well as against MSPDBL1 and MSPDBL2 inhibit parasite growth *in vitro* (17, 21–24). In addition, antibodies against MSP3, MSPDBL1, and MSPDBL2 are also involved in opsonizing merozoites for clearance by phagocytosis (24, 25). Antibodies against peripheral MSPs (MSP3, MSP6, MSPDBL1, and MSPDBL2) have also been shown to be involved in the antibody-dependent cellular inhibition mechanism, inhibiting parasite growth *in vitro* with the co-operation of blood monocytes (26–30). Because of the exposure of MSPs to the host immune system, several of these MSPs, such as MSP3, MSPDBL1, and MSPDBL2, have similar patterns of balancing selection where alleles have more intermediate frequen-

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² The abbreviations used are: MSP, merozoite surface protein; GPI, glycosylphosphatidylinositol; PEM, parasitophorous vacuole-enclosed merozoite; DBL, Duffy-binding-like.

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cies than expected in the absence of selection to maintain different alleles within populations (31–34). In particular, the erythrocyte binding proteins MSPDBL1 and MSPDBL2 have been identified to be the most polymorphic antigens in the *Plasmodium falciparum* population, with most polymorphisms occurring in the DBL domain, suggesting that these molecules are under high selection pressure from the host immune system (35). Together, MSPs appear to play significant roles in invasion, although the exact function and mechanism through which these proteins act during invasion have not been clearly described.

In this study, we use parasite-derived merozoite surface complexes to show that there are a variety of MSP1 complexes of differing sizes on the merozoite surface. We also show that there are overlapping functional roles for these complexes, with at least three MSP1 complexes that are able to bind directly to the erythrocyte surface. In addition, we describe a monoclonal antibody that targets the p83 fragment of MSP1, which has the ability to inhibit parasite growth *in vitro*. This inhibitory antibody targets the different forms of the MSP1 complex, providing evidence that targeting critically important regions of the core of these complexes remains a viable option for blocking invasion of *P. falciparum*.

Experimental Procedures

Ethics Statement—Antibodies were raised in mice and rabbits under the guidelines of the National Health and Medical Research Committee and the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The specific details of our protocol were approved by the Royal Melbourne Hospital Animal Welfare Committee under approval number AEC2011.009. The use of human blood was approved by the Walter and Eliza Hall Institute of Medical Research Human Ethics Committee under approval number HREC86/17.

Parasites—The 3D7 parental line and four knockout parasite lines (3D7 Δ MSP3, 3D7 Δ MSP6, 3D7 Δ MSPDBL1, and 3D7 Δ MSPDBL2) were used throughout this study (17, 23, 36). The *misp3*, *misp6*, *mispdbl1*, and *mispdbl2* genes were excised from 3D7 parasites individually. The pCC1 plasmid is flanked by the gene of interest as well as the human *dihydrofolate reductase* gene that confers resistance to the drug, WR99210. The knockout parasite lines were selected and maintained in the presence of 5 nM WR99210. Parasites were maintained in human erythrocytes (blood group O) at a hematocrit of 4% in the presence of 10% Albumax. To harvest parasite material at different time points, cultures were synchronized with sorbitol and allowed to grow to the specific time point before passing through a magnet to remove the majority of free red blood cells. Parasites were centrifuged at 1500 \times g for 5 min and treated with saponin. Parasite proteins were extracted from saponin-lysed material in the presence of 0.1% Triton X-100 on ice for 10 min before centrifugation at 10,000 \times g for 10 min with the addition of Complete protease inhibitors (Roche Applied Science) to minimize nonspecific proteolysis. Both the supernatant and pellet were harvested for SDS-PAGE and Western blotting. To harvest invasion supernatant, synchronized late schizont stage parasites were allowed to rupture and reinvade

red blood cells before culture medium was harvested post-invasion by centrifugation at 10,000 \times g for 10 min.

Immunofluorescence Assay—Trophozoites (28–32 h), schizonts (44–48 h), and PEM (E64-treated) parasites were smeared, acetone/methanol-fixed, and blocked in PBS, 3% BSA for 1 h. Primary antibodies were diluted in PBS, 3% BSA at 1:100 dilution and incubated on slides for 2 h at 25 °C before washing in PBS. Secondary antibodies labeled with 488 or 594 fluorophores were incubated at 1:300 dilution for 30 min, washed in PBS, and air-dried. The nuclei of the parasites were stained with DAPI nuclear stain at 0.2 μ g/ml in Vectashield to prevent photobleaching. Images were captured and deconvoluted on a DeltaVision Elite microscope at \times 100 magnification.

Immunoblotting and Antibodies—Proteins were separated by 4–12% BisTris SDS-PAGE (Invitrogen). Standard Western blotting procedures were performed using a nitrocellulose membrane (0.45 μ m), and the immunoblots were processed with ECL substrates (Pierce). For all Western blots containing parasite material and recombinant proteins, antibodies were used at a 1:2000 dilution. With the exception of RMAL302 and RMAL8 antibodies that were raised against wheat germ cell-free-produced, full-length MSP3 and MSP7, respectively (Kitayama Labes, Ina, Japan), the remaining antibodies were raised against recombinantly produced *Escherichia coli* MSP proteins as described below. Antibodies used to detect MSP1 were anti-MSP1 monoclonal 9A6 antibodies targeting the p83 fragment, anti-MSP1 monoclonal 5H7 antibodies targeting the p38 fragment, anti-MSP1 monoclonal 8D4 antibodies targeting the p30 fragment, and anti-MSP1 rabbit polyclonal R1566 antibodies. MSP3 was detected using anti-MSP3 monoclonal 9A12 or anti-MSP3 polyclonal RMAL302 antibodies. MSP6 was detected using anti-MSP6 monoclonal 2C12 or anti-MSP6 polyclonal R1467 antibodies. MSPDBL1 was detected using anti-MSPDBL1 monoclonal 7H12 or anti-MSPDBL1 polyclonal R1277 antibodies. MSPDBL2 was detected using anti-MSPDBL2 monoclonal 4A7 or anti-MSPDBL2 polyclonal R1296 antibodies. MSP7 was detected using anti-MSP7 monoclonal 8F1 or RMAL8 antibodies.

Immunoprecipitation of Complexes from Invasion Supernatant—The invasion supernatant was concentrated 20-fold, where 1 ml of concentrate was equivalent to a 20 ml of culture. 200 μ l of concentrated invasion supernatant was allowed to bind to 5 μ g of monoclonal antibody for 1 h at 25 °C. 50 μ l of Sepharose G resin was added to the mixture and loaded onto an empty microspin column. The resin was washed with 5 ml of PBS and 0.4% Triton X-100. The resin was then eluted with 50 μ l of 1 \times reducing sample buffer (RSB) for analysis on SDS-PAGE and immunoblot.

Expression of Recombinant MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 Constructs in Escherichia coli—Codon-optimized synthetic genes (GeneArt) were generated (MSP3_{191–354}, MSP6_{161–371}, MSPDBL1_{105–697}, MSPDBL2_{127–762}, and MSP7_{177–351}) and digested with BamHI and XhoI before ligating into the pPROEXHTb vector containing an N-terminal His₆ tag (Invitrogen). MSP1 was expressed and purified as described previously (16). Other recombinant proteins were also expressed and purified as described previously (16, 17, 37, 38).

Recombinant MSP Co-immunoprecipitation—Recombinant proteins at 2 μg each were allowed to incubate for 3 h at 25 °C in a volume of 50 μl . The resulting mixture was then added to 5 μg of monoclonal antibody in a final volume of 200 μl . Following a 1-h incubation, 50 μl of Sepharose G was added for a further 1 h. Post-incubation, the mixture was centrifuged and washed twice with PBS-Tween 20 (PBS-T) before eluting with sample buffer supplemented with β -mercaptoethanol. The proteins were analyzed by SDS-PAGE and Western blotting to positively identify interacting partners.

ELISAs—Plates were coated overnight at 4 °C with 100 μl of recombinant MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, or MSP7 at a concentration of 2 $\mu\text{g}/\text{ml}$. Wells were washed three times in PBS-Tween 20 before blocking for 1 h at 25 °C in 5% skim milk/PBS. Following blocking, anti-MSP1, -MSP3, -MSP6, -MSPDBL1, -MSPDBL2 and -MSP7 monoclonal antibodies in 5% skim milk/PBS were allowed to incubate for 1 h at 25 °C. The wells were washed three times with PBS-Tween 20 and twice with PBS before 100 μl of peroxidase substrate was added and allowed to incubate for 4 min before the reaction was stopped with 100 μl of 1 M phosphoric acid. The absorbance at 450 nm was collected and tabulated for analysis. Each ELISA was set up in duplicate, and results represent three independent assays. Competition ELISAs were performed by coating the plates with 100 μl of recombinant MSP3, MSP6, MSPDBL1, MSPDBL2, MSP7, and AMA1 at a concentration of 1 $\mu\text{g}/\text{ml}$. Following washing and blocking as described above, 100 μl of MSP1 preincubated with anti-MSP1 monoclonal 9A6 antibody at 1 $\mu\text{g}/\text{ml}$ was added to the plate and allowed to incubate for 1 h. The wells were washed, and anti-MSP1 rabbit polyclonal antibody was added for 1 h before the addition of goat anti-rabbit HRP secondary antibodies. The ELISAs were developed as described.

Separation of Complexes—3D7 invasion supernatant was harvested at 20% rings and concentrated 20-fold using a 100-kDa molecular weight cutoff concentrator (Millipore) to remove non-complexed proteins. 400 μl of concentrated supernatant (~8 ml of culture) was loaded onto a Superose 6 10/300GL column (GE Healthcare) into PBS. Fractions were collected in 1-ml aliquots for analysis.

Erythrocyte Binding Assays—Fresh whole blood samples were obtained from the Australian Red Cross. 200 μl of concentrated invasion supernatant equivalent to 4 ml of culture was allowed to incubate with 50 μl of packed volume human RBCs in PBS for 2 h at 25 °C in a final volume of 500 μl . The mixture was passed through 400 μl of dibutyl phthalate (Sigma) and centrifuged. The supernatant was discarded, and the pellet was eluted in PBS with 1 M NaCl. The resulting supernatant after centrifugation was subjected to SDS-PAGE followed by immunoblotting. Erythrocyte binding assays using recombinant proteins were performed as described above using 2 μg of protein in a final volume of 300 μl with 50 μl of packed human erythrocytes. Enzyme treatments on erythrocytes were performed as follows. Washed human erythrocytes were incubated with chymotrypsin (1.5 mg/ml), trypsin (1.5 mg/ml), or neuraminidase (66.7 milliunits/ml) for 1 h at 37 °C before a soybean trypsin inhibitor was added to the enzyme-treated erythrocytes at 1.5 mg/ml. The treated erythrocytes were

washed three times with PBS before the concentrated invasion supernatant was allowed to bind as above.

Growth Inhibition Assay—Two cycle growth inhibition assays were performed where 2-fold serial dilutions of monoclonal antibodies, starting at 1 mg/ml final concentration, was added to late-stage *P. falciparum* cultures at 0.15% parasitemia in 2% hematocrit in 96-well round bottom microtiter plates. After incubation for 2 cycles (96 h) of growth, the parasitemia for each individual well was determined by flow cytometry (FACSCalibur 1). Parasitemia was expressed as a percentage of growth compared with a non-inhibitory antibody control.

Results

Processed Peripheral Merozoite Surface Proteins Are Incorporated onto the Surface of Merozoites Late in Schizogony—MSPs are processed during schizogony (Fig. 1A). 3D7 parasites were harvested at various stages of development during schizogony. Trophozoites (28–32 h), late schizonts (44–48 h), and parasitophorous vacuole-enclosed merozoites (PEMs) and all merozoite surface proteins tested (MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7) were expressed in schizonts and PEMs but not in trophozoites (Fig. 1B). To determine whether peripheral MSPs are processed upon incorporation onto the surface of merozoites or in the parasitophorous vacuole, parasites were treated with saponin and separated into a supernatant fraction, containing proteins from the parasitophorous vacuole, and a pellet fraction, with those from the parasite surface. AMA1, a type I integral membrane protein that is expressed in segmenting schizonts, was used as a control (39–41). Both the 83-kDa precursor and 66-kDa processed fragments were found attached to the merozoite surface in late-stage schizonts and PEMs but not shed in the supernatant after invasion (Fig. 1C). In contrast, the surface coat of merozoites is shed during invasion into the culture supernatant. The merozoite surface proteins MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 (Fig. 1A) were confirmed to be in the culture supernatant, and they showed similar expression profiles during parasite development, with levels peaking late in schizogony (44–48 h) (Fig. 1, B and C). As expected, MSP1, which has a GPI anchor, was detected associating exclusively on the parasite surface, as indicated by its location in the pellet fraction (Fig. 1C). The processing of the various MSP1 fragments was visualized using specific antibodies targeting p83, p38, or p30 of MSP1, and the expected bands were observed as described previously for proteolytic processing of this protein by PfSub1 during schizogony (Fig. 1, A and C) (42).

MSP3 processing occurs before incorporation onto the surface of merozoites, as evident from the 50- and 44-kDa fragments of MSP3 detected in the supernatant fraction (Fig. 1C). In contrast, the peripheral proteins MSP6, MSPDBL1, and MSPDBL2 were predominantly found in the supernatant fraction in late schizonts, with only a small proportion observed in the pellet fraction (Fig. 1C). This was consistent with them being released into the parasitophorous vacuole prior to association with the merozoite surface. As the schizonts developed, the peripheral MSPs became associated with the merozoite surface, as evidenced by the increased intensity of bands in the pellet fraction of the PEM preparation compared with the pellet

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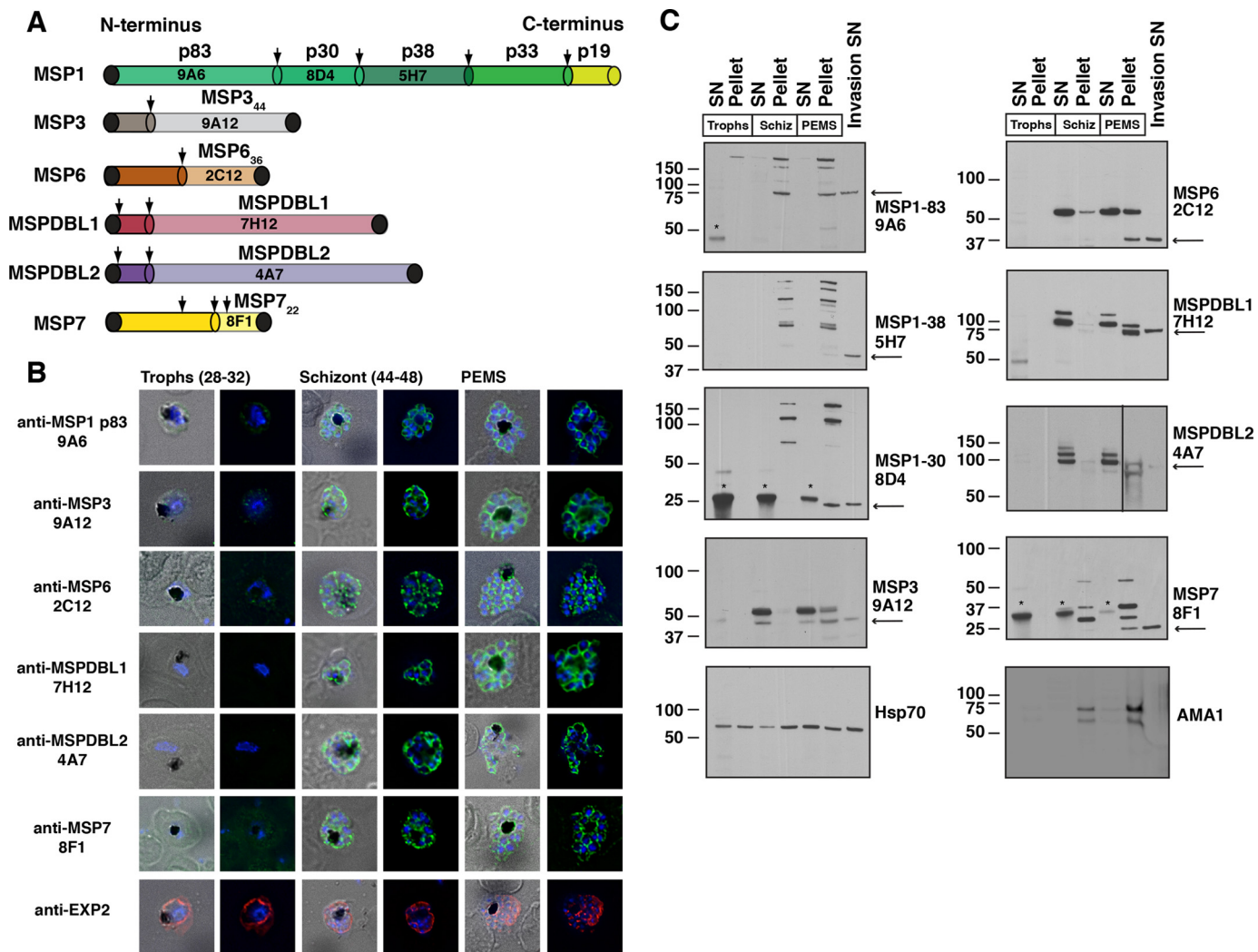


FIGURE 1. Processed peripheral MSPs become part of an anchored complex late in schizogony. *A*, schematic showing the primary structure and processing sites of MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7, with the final shed products indicated above the protein. Arrows indicate processing sites. Monoclonal antibodies with epitopes that target specific domains within the MSPs are shown (*i.e.* 9A6 (anti-MSP1 p83), 8D4 (anti-MSP1 p38), 5H7 (anti-MSP1 p30), 9A12 (anti-MSP3), 2C12 (anti-MSP6), 7H12 (anti-MSPDBL1), 4A7 (anti-MSPDBL2), and 8F1 (anti-MSP7)). *B*, immunofluorescence assays of trophozoites (Trophs, 28–32 h) in columns 1 (phase + 488 + DAPI) and 2 (488 + DAPI), schizonts (Schiz, 44–48 h) in columns 3 (phase + 488 + DAPI) and 4 (488 + DAPI), and PEMS (E64-treated) in columns 5 (phase + 488 + DAPI) and 6 (488 + DAPI) were probed with the monoclonal antibodies (green) 9A6 (anti-MSP1 p83), 9A12 (anti-MSP3), 2C12 (anti-MSP6), 7H12 (anti-MSPDBL1), 4A7 (anti-MSPDBL2), and 8F1 (anti-MSP7). EXP2 was used as an expression control probed with polyclonal rabbit anti-EXP2 antibodies (red). The nuclei of parasites were stained with DAPI (blue). *C*, parasites were harvested at three stages (trophozoites (28–32 h), schizonts (44–48 h), and PEMS (E64-treated)) together with the supernatant (SN) from invaded merozoites. Timing of processing and incorporation of MSPs onto a GPI-anchored MSP complex was observed through immunoblots of the three parasite stages, containing the supernatant fraction, which represents proteins that are in the parasitophorous vacuole, and the pellet fraction (represents proteins on the merozoite surface). The culture supernatant was released into the medium during merozoite invasion. Immunoblots for MSP1 p83, MSP1 p38, MSP1 p30, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 are shown, along with the monoclonal antibodies used, together with Hsp70 and AMA1, which is an expression control. Asterisks indicate nonspecific bands from red blood cell contaminants in the supernatant control, and arrows indicate the final processed products of the various MSPs.

fraction from schizonts (Fig. 1C). The final 36-, 75-, and 90-kDa processed products of MSP6, MSPDBL1, and MSPDBL2, respectively, were exclusively with the parasite membrane and not observed in the supernatant fraction throughout schizogony, indicating that the final proteolytic event occurred after these MSPs were incorporated onto the parasite surface as a complex (Fig. 1C).

Previously, it has been shown that MSP7 and MSP1 come together in a pre-Golgi compartment (37), and, in late schizonts, these proteins were found in the membrane-associated fraction despite MSP7 lacking either a transmembrane domain or a GPI anchor (Fig. 1C) (43). The processed 22-kDa MSP7 fragment was first observed in the PEM pellet, indicating that

the final processing event happens very late into schizogony while it is in complex with MSP1 (Fig. 1C).

In summary, these results show that the peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 have a complex processing pattern that varies in time for the different proteins with respect to merozoite development. This is likely to be important for their correct assembly into the MSP1 complex on the merozoite surface.

The Peripheral Merozoite Surface Proteins MSP3, MSP6, MSPDBL1, MSPDBL2 and MSP7 Form a Complex with MSP1 on the Merozoite Surface—To confirm that MSP1 is the platform for these peripheral MSPs to associate with the merozoite, immunoprecipitation assays were performed using anti-MSP1

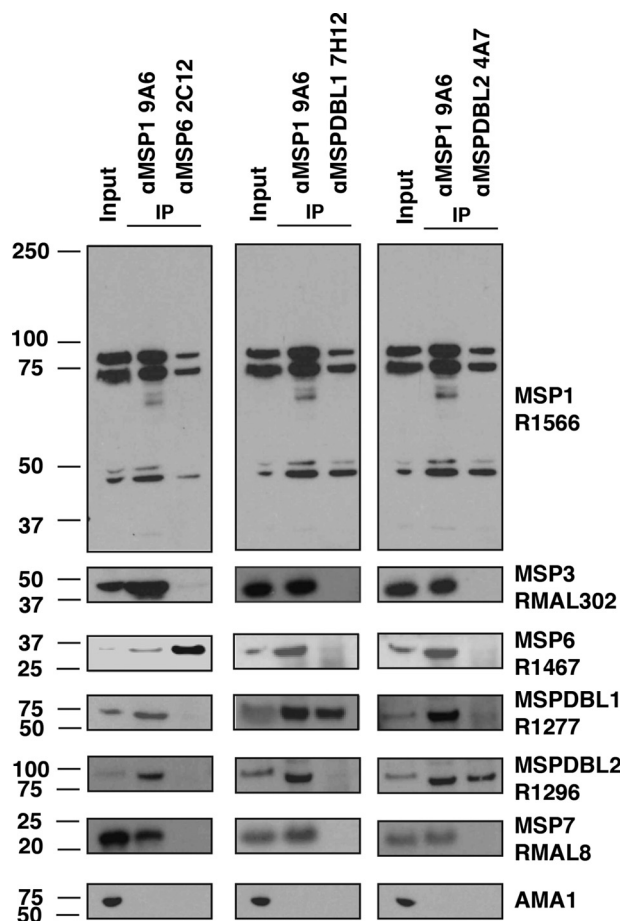


FIGURE 2. MSP1 is the platform on the merozoite surface with which other MSPs interact. 3D7 parental parasite invasion supernatant was immunoprecipitated with monoclonal antibodies (9A6 (anti-MSP1), 2C12 (anti-MSP6), 7H12 (anti-MSPDBL1), or 4A7 (anti-MSPDBL2)). The first lane shows the invasion supernatant (*Input*) along with the eluates from immunoprecipitations (*IP*) of anti-MSP1, anti-MSP6, anti-MSPDBL1, or anti-MSPDBL2. The immunoblots were probed with the rabbit polyclonal antibodies R1566 (anti-MSP1), RMAL302 (anti-MSP3), R1467 (anti-MSP6), R1277 (anti-MSPDBL1), R1296 (anti-MSPDBL2), or RMAL8 (anti-MSP7). AMA1 was used as a negative control.

monoclonal antibodies. Anti-MSP1 antibodies immunoprecipitated MSP1 from invasion supernatant as well as the peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 (Fig. 2). To determine whether these MSPs were present in the same complex, monoclonal antibodies specific for MSP6, MSPDBL1, and MSPDBL2 were used to immunoprecipitate the same culture supernatant. An anti-MSP6 monoclonal antibody immunoprecipitated only MSP1 and MSP6 but not MSP3, MSPDBL1, MSPDBL2, or MSP7 (Fig. 2). In contrast, anti-MSPDBL1 and anti-MSPDBL2 monoclonal antibodies only immunoprecipitated the corresponding protein. This was consistent with the presence of MSP6, MSPDBL1, and MSPDBL2 in independent MSP1 complexes and provides evidence that there are multiple MSP1 complexes present on the merozoite surface.

Recombinant proteins for the peripheral MSPs were used to confirm that these proteins interact directly with MSP1. Recombinant MSP1 consisting of the p83/p30 (102-kDa) and p38/p42 (89.7-kDa) domains were purified as a complex and used to confirm the interaction of recombinant MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 that represent the final

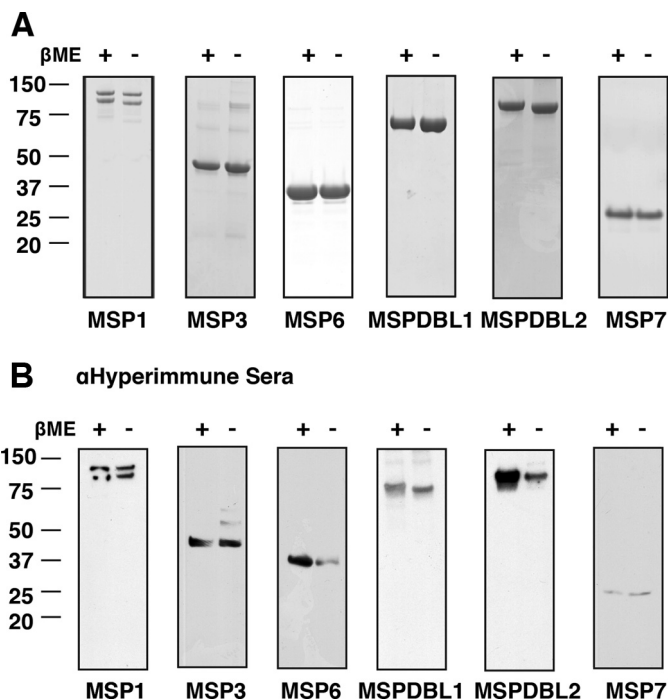


FIGURE 3. Recombinant MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 are recognized by human sera. *A*, final purified recombinant proteins for MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 are shown on a Coomassie-stained SDS-PAGE in the presence (+) and absence (–) of β -mercaptoethanol (β ME). *B*, the recombinant proteins were probed on an immunoblot with pooled sera obtained from individuals infected by malaria in highly endemic regions in Papua New Guinea (*αHyperimmune Sera*).

mature form of these proteins found in shed complexes (Fig. 3A). These recombinant proteins were recognized by sera obtained from individuals who had been exposed to malaria (Fig. 3B). To obtain reagents specific for each protein, we made mouse monoclonal antibodies and identified those that recognized the expected processed product in culture supernatant and the corresponding recombinant proteins using immunoblots (Fig. 4). Additionally, the monoclonal antibodies only recognized the specific recombinant protein in ELISA experiments (Fig. 4). Recombinant MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 were incubated with the MSP1 complex and co-immunoprecipitated with each specific monoclonal antibody. Anti-MSP1 antibodies were able to immunoprecipitate MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 separately (Fig. 5A). Likewise, reciprocal immunoprecipitation experiments showed that anti-MSP3, anti-MSP6, anti-MSPDBL1, anti-MSPDBL2, and anti-MSP7 antibodies were also able to pull down the MSP1 complex (Fig. 5A). AMA1 was used as a control to show that the immunoprecipitations were specific (Fig. 5B). Accordingly, recombinant MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 bind directly and individually to the p83/p30 and p38/p42 MSP1 complex.

MSP1 Exists as Complexes of Different Sizes and Components—To further characterize the different MSP1 complexes in culture supernatant, they were separated by size exclusion chromatography, and the fractions were analyzed (Fig. 6). The main peaks were ~66 kDa and 132 kDa and likely correspond to monomeric and dimeric BSA derived from the parasite culture medium (Fig. 6A). Each fraction starting from

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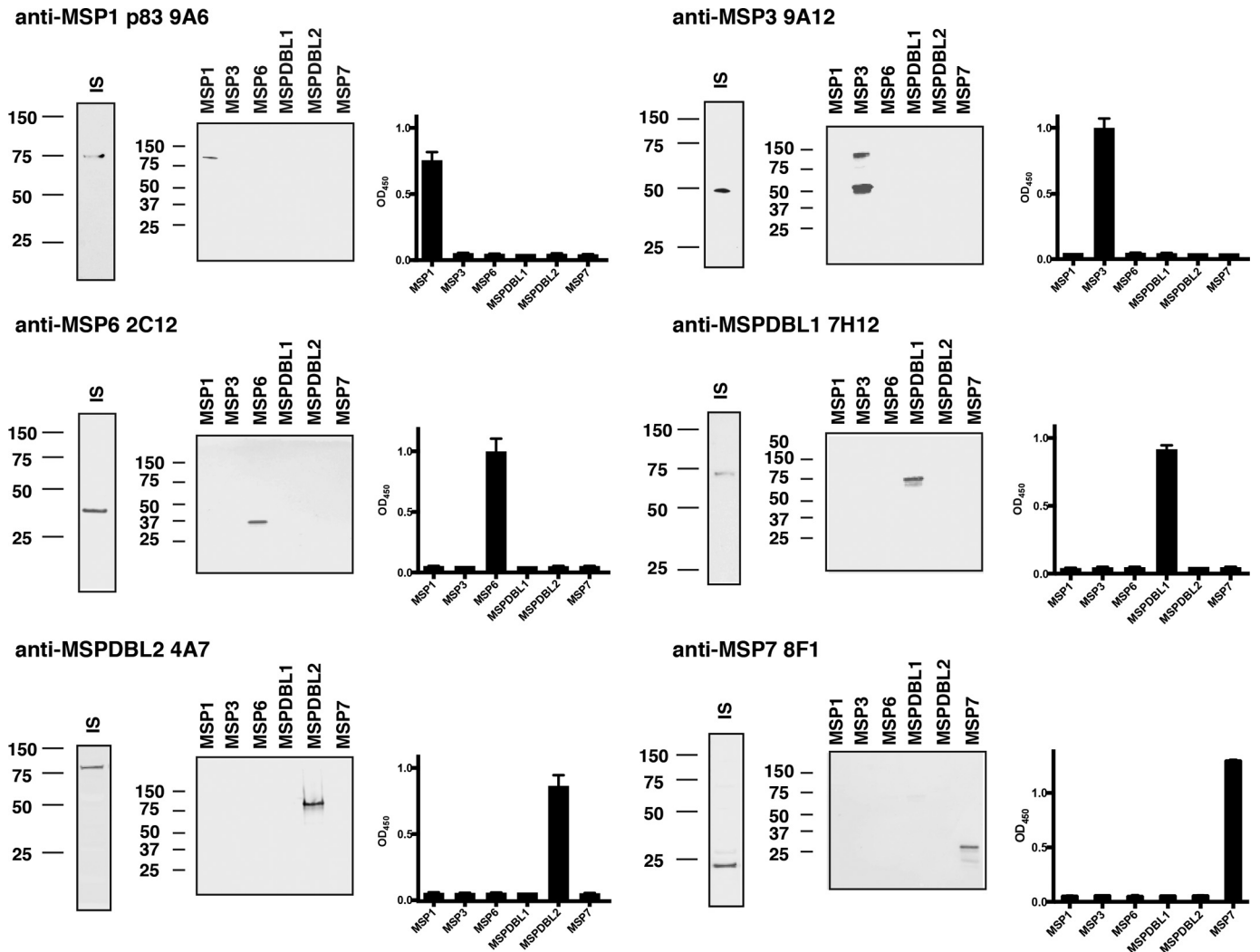


FIGURE 4. The monoclonal antibodies against MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 used for immunoprecipitation assays are specific. Monoclonal antibodies against MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, MSP7, and MSP9 were screened for their specificity against native invasion complexes from parasites (left panels) and recombinant proteins (center panels) using immunoblots. The monoclonal antibodies were also screened with ELISAs (right panels) to show specificity to the antigen of interest. *IS*, invasion supernatant.

the void volume of the column was probed separately with antibodies to MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7. MSP1 was found in a broad number of fractions, consistent with the presence of a number of different complexes made of different constituents (Fig. 6B). In comparison, the peripheral MSPs that bind to MSP1 showed very different elution profiles. MSP3 eluted between fractions 7–12, MSP6 between 8–11, MSPDBL1 between 4–8, MSPDBL2 between 11–14, and MSP7 between 8–10 (Fig. 6B). These elution profiles suggest that differently sized complexes may exist on the parasite surface. There was a large spread across the estimated size of these complexes, ranging from approximately >670 to 100 kDa. The MSP1-MSPDBL1 complex eluted much earlier than the 670-kDa standard and was estimated to be megadaltons in size (Fig. 6C). In comparison, the MSP1-MSP3 complex eluted at ~400 kDa, the MSP1-MSP6 complex at ~200 kDa, the MSP1-MSPDBL2 complex at ~150 kDa, and the MSP1-MSP7 complex at ~250 kDa. To confirm that, following size fractionation, the peripheral MSPs were still present in the form of a complex, each fraction corresponding from 4–14 was subjected to

immunoprecipitation with an anti-MSP1 antibody. All peripheral MSPs were co-immunoprecipitated with MSP1, indicating that these MSPs were still in complex with MSP1 (Fig. 6D).

MSP Complexes Containing MSP1, MSP6, MSPDBL1, and MSPDBL2 Bind to Red Blood Cells—MSP1, or components within the MSP1 complex, have been reported to mediate binding of the merozoite to erythrocytes. To evaluate which of these MSP1 complexes can bind to erythrocytes, culture supernatant was incubated with RBCs, and bound complexes were subsequently eluted and analyzed. Complexes that contained MSP1, MSP6, MSPDBL1, and MSPDBL2 were able to bind directly to erythrocytes (Fig. 7A). In comparison, complexes containing MSP3 and MSP7 were unable to bind to erythrocytes, suggesting a role independent of erythrocyte binding for these complexes. Erythrocyte binding of gel filtration-separated fractions was also assessed to determine whether size fractionation affected erythrocyte binding. Similar to the binding profile of the culture supernatant, complexes containing MSP1, MSP6, MSPDBL1, and MSPDBL2, but not MSP3 and MSP7, were able to bind to red blood cells (Fig. 7B).

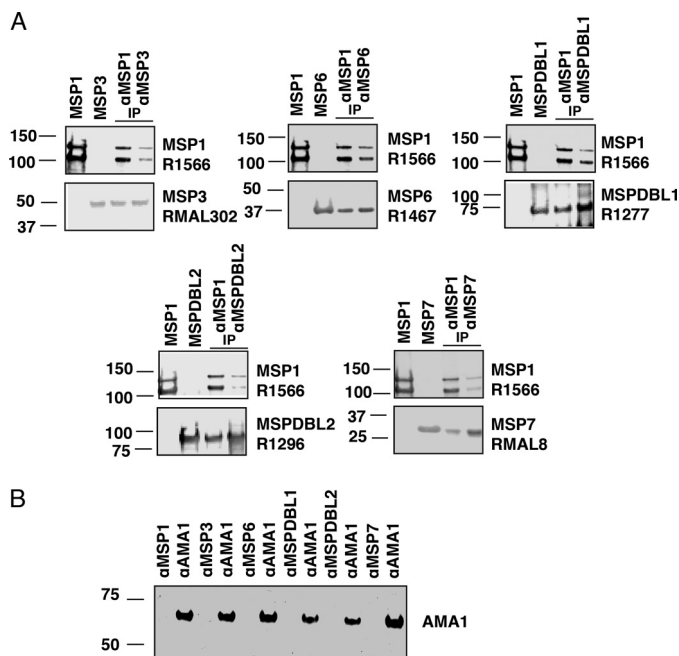


FIGURE 5. Recombinant MSPs interact with MSP1. *A*, recombinant proteins were incubated with recombinant MSP1 and immunoprecipitated (IP) with either anti-MSP1 monoclonal antibody or each of the monoclonal antibodies specific for MSP3, MSP6, MSPDBL1, MSPDBL2, or MSP7. The first and second lanes of each immunoblot represent the input of each recombinant protein and demonstrate that the antibodies are not cross-reactive. *B*, recombinant AMA1 immunoprecipitated with anti-AMA1 antibodies but did not co-immunoprecipitate with MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, or MSP7.

Erythrocytes were treated with trypsin, chymotrypsin, and neuraminidase to preferentially remove receptor components from the cell surface. The enzyme treatment binding profiles for MSPDBL1 and MSPDBL2 have been shown previously to be insensitive to chymotrypsin, trypsin, and neuraminidase treatments (17, 19). MSP1 complexes that bound erythrocytes also showed a chymotrypsin, trypsin, and neuraminidase treatment-resistant phenotype consistent with binding through MSPDBL1 and MSPDBL2. The MSP1-MSP6 complex, which does not include either MSPDBL1 or MSPDBL2, also showed an ability to bind to erythrocytes, and this was also resistant to chymotrypsin, trypsin, and neuraminidase treatment of the host cell (Fig. 7C). In contrast, EBA175, which binds to Glycophorin A, was sensitive to both trypsin and neuraminidase treatment of erythrocytes as expected (44, 45). Although it is not possible to identify specific receptors on the erythrocyte, these data confirm that the MSP1-MSP6 complex can bind erythrocytes and that the receptor(s) has/have similar properties as that which binds the MSP1-MSPDBL1 and MSP1-MSPDBL2 complexes.

To delineate the erythrocyte binding proteins within the MSP1 complexes, erythrocyte binding assays were performed on recombinant proteins corresponding to the mature forms of MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 (Fig. 3A). Of the six recombinant proteins tested, only recombinant MSPDBL1 and MSPDBL2 bound to red blood cells (Fig. 7D), consistent with previous studies (17). Interestingly, although native parasite complexes containing MSP6 bound to erythrocytes, the MSP6₃₆ fragment alone did not. This suggests that the MSP1 fragments within this complex, or potentially a third

unidentified merozoite surface protein that is within the complex, interacts directly with the host cell.

Other MSP1 Complexes Are Not Disrupted When Single Genes of Peripheral MSPs Are Deleted—Single gene knockouts of *mSP3*, *mSP6*, *mSPDBL1*, and *mSPDBL2* were generated, and lines were established that did not express MSP3, MSP6, MSPDBL1, or MSPDBL2, respectively (Fig. 8A). Although single gene deletions did not affect parasite viability, it is currently unknown whether deletion of one component affects the formation of other MSP1 complexes. To address this, MSP1 complexes were immunoprecipitated from 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 parasites. We observed that the deletion of *mSP3*, *mSP6*, *mSPDBL1*, or *mSPDBL2* was insufficient to disrupt other MSP1 complexes (Fig. 8A). For example, in 3D7ΔMSP3 parasites, the MSP1-MSP6, MSP1-MSPDBL1, and MSP1-MSPDBL2 complexes were not affected and were able to be co-immunoprecipitated with MSP1. Similarly, when either the *mSP6*, *mSPDBL1*, or *mSPDBL2* gene was deleted, the other erythrocyte binding complexes were still present and could be co-immunoprecipitated with MSP1 (Fig. 8A). We next tested whether gene deletions impaired the ability of other MSP1 complexes to bind to erythrocytes (Fig. 8B). Deleting the *mSP6* gene had no effect on the ability of the MSP1-MSPDBL1 and MSP1-MSPDBL2 complexes to bind to red blood cells. Similarly, deleting the *mSPDBL1* or *mSPDBL2* gene had no effects on the ability of the MSP1-MSP6 and MSP1-MSPDBL2 or MSP1-MSP6 and MSP1-MSPDBL1 complexes, respectively, to bind erythrocytes. This suggests that multiple MSP1 complexes have overlapping functional roles and are able to compensate when one form of the complex is blocked or disrupted.

Inhibitory Monoclonal Antibodies against MSP1 p83 Inhibit Parasite Growth—The monoclonal antibodies used in this study were tested to determine whether they could interfere with erythrocyte invasion and subsequently inhibit the growth of *P. falciparum*. Of the eight monoclonal antibodies (9A6, 8D4, 5H7, 9A12, 2C12, 7H12, 4A7, and 8F1), only the MSP1 p83 monoclonal antibody 9A6 showed a growth inhibition of up to 40.46% ± 5.977% ($p < 0.0001$) at 1 mg/ml compared with a non-inhibitory MSPDBL2 monoclonal antibody of the same IgG isotype following two blood stage cycles (Fig. 8C). To determine whether the p83 monoclonal antibody targets particular MSP1 complexes, it was tested against 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2, which represent complexes that bind to erythrocytes, and the 3D7ΔMSP3 line, which does not bind to red blood cells. Each of the gene knockout lines showed increasing levels of inhibition with 2-fold increases between 0.03125 and 1 mg/ml of antibodies (Fig. 8D). Compared with a non-inhibitory IgG control at 1 mg/ml, significant levels of inhibition were observed using anti-MSP1 p83 monoclonal antibodies (Fig. 8E). There was a decrease in parasite growth by 40.68% ± 7.004% ($p = 0.0001$), 30.47% ± 8.183% ($p = 0.0047$), 33.68% ± 5.382% ($p < 0.0001$), and 34.28% ± 5.252% ($p < 0.0001$) for the 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 lines, respectively (Fig. 8E). Additionally, there was no distinguishable difference in growth between each knockout line and the parental line at the highest antibody concentration, suggesting that the monoclo-

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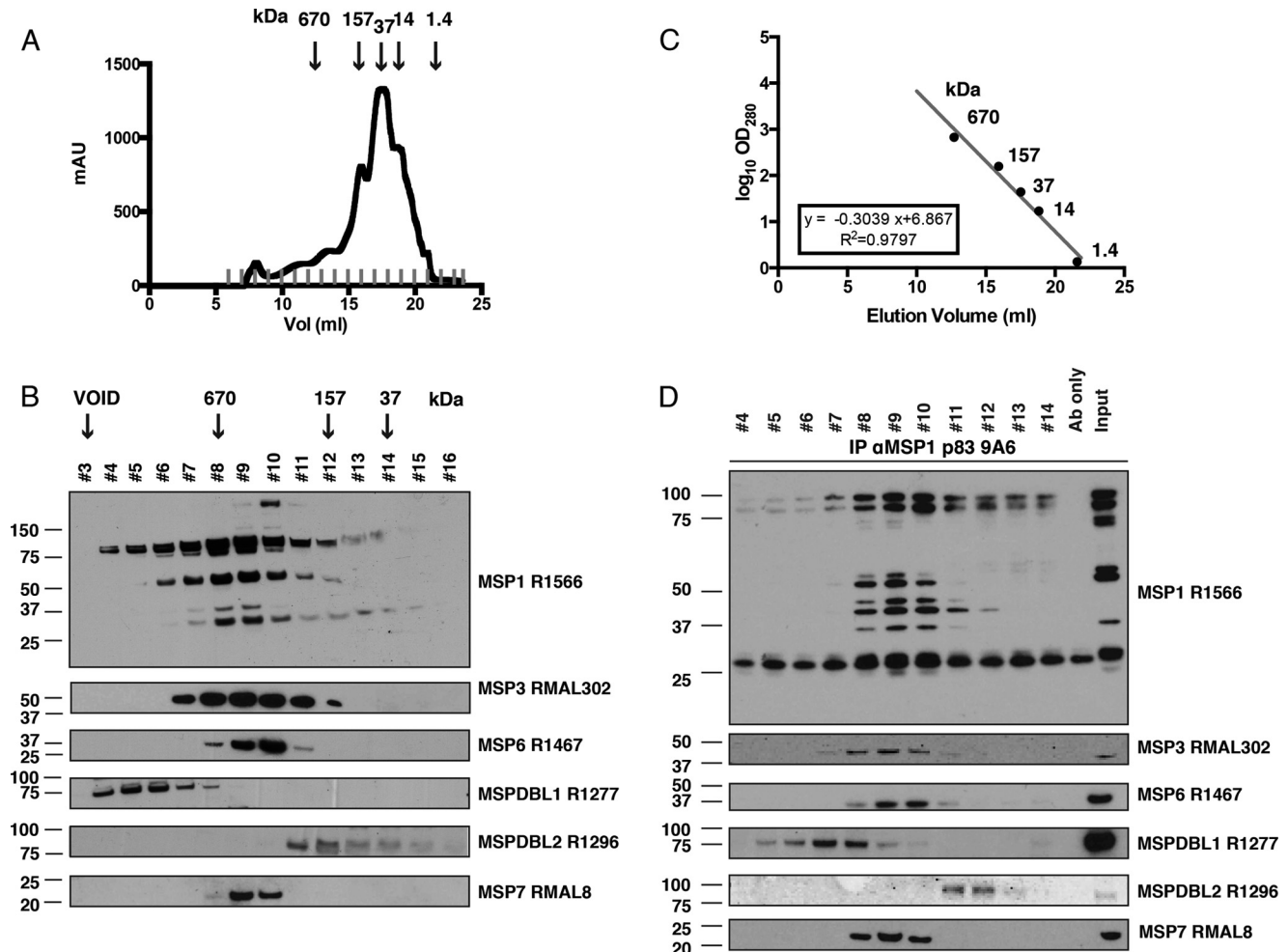


FIGURE 6. **MSP1 complexes vary in size.** *A*, gel filtration profile of concentrated invasion supernatant on Superose 6 10/300 GL with estimated molecular masses indicated by arrows. *mAu*, milli-absorbance units. *B*, immunoblots of Superose 6-fractionated invasion supernatant show that proteins MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 elute in different fractions at very high molecular masses. Molecular masses of standards (thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B₁₂ (1.4 kDa)) with elution volumes of 12.70, 15.92, 17.52, 18.83, and 21.59 ml, respectively. Approximate molecular mass was fitted to $y = -0.3039x + 6.867$, where $R^2 = 0.9797$. *D*, Superose 6-fractionated samples 4–14 were immunoprecipitated (IP) with an anti-MSP1 monoclonal antibody (Ab), and eluates were probed with antibodies against MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7.

nal antibody targeted all MSP1 complexes present on the parasite surface (Fig. 8E). Competition ELISAs were performed to further understand whether the anti-MSP1 p83 9A6 antibody functions to block complex formation. In the presence of this monoclonal antibody, recombinant MSP3, MSP6, MSP7, MSPDBL1, and MSPDBL2 were still able to bind to MSP1 at similar levels compared with its binding in the absence of 9A6 (Fig. 8F). This suggests that this anti-MSP1 p83 antibody inhibits parasite growth in a manner independent of blocking complex formation.

Discussion

MSPs are implicated in the initial stages of invasion of *P. falciparum* into human erythrocytes. The most abundant MSP on the parasite surface is MSP1, and it plays an essential role for parasite survival (1). Recent work has shown that the processing of MSP1 not only produces mature MSP1 fragments important for parasite viability but also appears to be important for parasite egress (46). Although the precise role of MSP1 during invasion has not been described, it is known that processed MSP1

binds to multiple peripheral merozoite surface proteins to form large macromolecular structures on the parasite (16, 17, 47). However, the description and characterization of these macromolecular structures have been incomplete, and the functional role of MSP1 complexes has not been fully evaluated. In this study, we have shown that multiple complexes of varying sizes exist on the parasite surface, where peripheral MSPs bind to MSP1 that function as a platform for them to be presented on the merozoite surface. At least three of these MSP1 complexes bind to erythrocytes, and they appear to have overlapping functional roles in invasion.

Many *P. falciparum* proteins, including MSPs, are modified extensively during schizogony through sequential proteolytic events mediated by proteases to ensure successful invasion of the merozoite into human erythrocytes (48). In addition, the peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 do not have a transmembrane domain or GPI anchor and therefore interact with anchored proteins to be presented on the merozoite surface. We observed that MSP3, MSP6, MSPDBL1, and MSPDBL2 expression levels

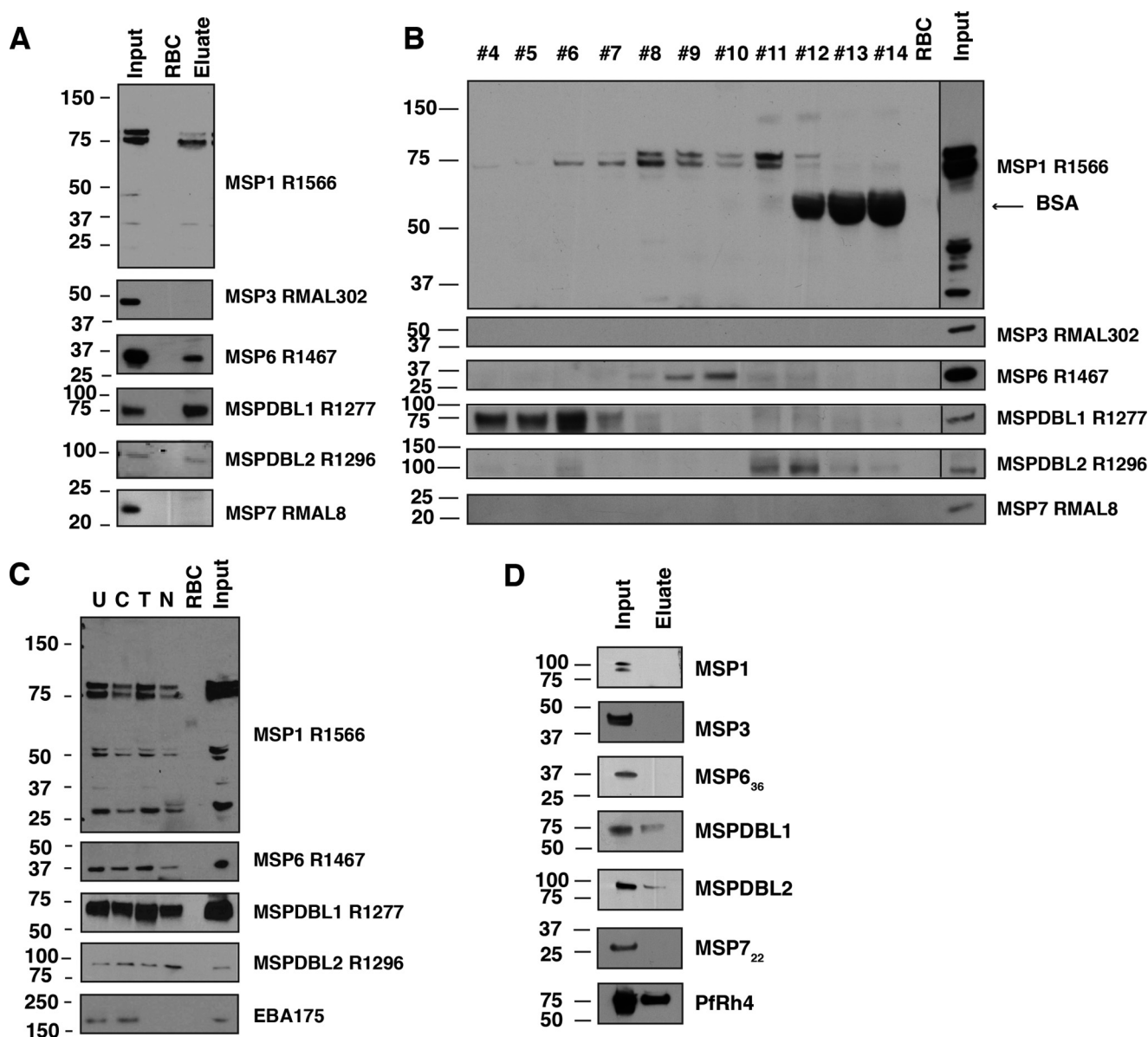


FIGURE 7. MSP1 complexes have different roles in invasion. *A*, invasion supernatants harvested were allowed to bind to red blood cells, and the eluates were immunoblotted with specific antibodies against MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7. The *first lane* represents the invasion supernatant input, the *second lane* is an RBC control that detects any nonspecific reactivity of antibodies to red blood cells, and the *third lane* is the eluate from the red blood cell binding assay. *B*, Superose 6-fractionated invasion supernatant (fractions 4–14) were incubated with host RBCs, and eluates were electrophoresed by SDS-PAGE and immunoblotted with either anti-MSP1, MSP3, MSP6, MSPDBL1, MSPDBL2, or MSP7 antibodies. PBS was used as an RBC control for control for nonspecific red blood cell proteins recognized by the antibodies. Proteins from invasion supernatant prefractionated served as molecular weight controls in the input lane. *C*, concentrated invasion supernatants were incubated with untreated RBCs (*U*) or RBCs treated with either chymotrypsin (*C*), trypsin (*T*), or neuraminidase (*N*). The complexes that contained MSP1, MSP6, MSPDBL1, and MSPDBL2 were able to bind to RBCs in a chymotrypsin-, trypsin-, and neuraminidase-resistant manner compared with EBA175, which binds in a trypsin- and neuraminidase-sensitive manner. *D*, recombinant proteins expressed in *E. coli* were subjected to binding to the RBC binding assay. Only recombinant MSPDBL1 and MSPDBL2 were able to bind directly to red blood cells. PfRh4 binds to complement receptor 1 on the red blood cell surface and served as a positive control.

peak late in schizogony and are first released into the parasitophorous vacuole of the parasite before being incorporated onto the merozoite surface. Following successful processing of these peripheral MSPs on the surface, a variety of MSP1 complexes that range in size between 150 and >670 kDa are formed (Fig. 9). Invasion ensues, and these complexes are shed into the supernatant following successful invasion, including MSP1 with the processed forms of MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7. The detection of only the processed fragments of these proteins in the shed complexes suggest that pro-

teolytic events preinvasion could be in part linked to the assembly and functional capacity of these complexes. It is not known whether specific inhibition of processing MSP3, MSP6, MSPDBL1, MSPDBL2, or MSP7 would block either their ability to assemble with MSP1 on the merozoite surface or impact their function during invasion. However, inhibition of PfSub2 processing of the MSP1 p42 fragment using inhibitory antibodies blocks the release of the complex from the merozoite surface which, in turn, halts invasion (48, 49). In addition, PfSub2 action is prevented by antibodies targeting a variety of epitopes

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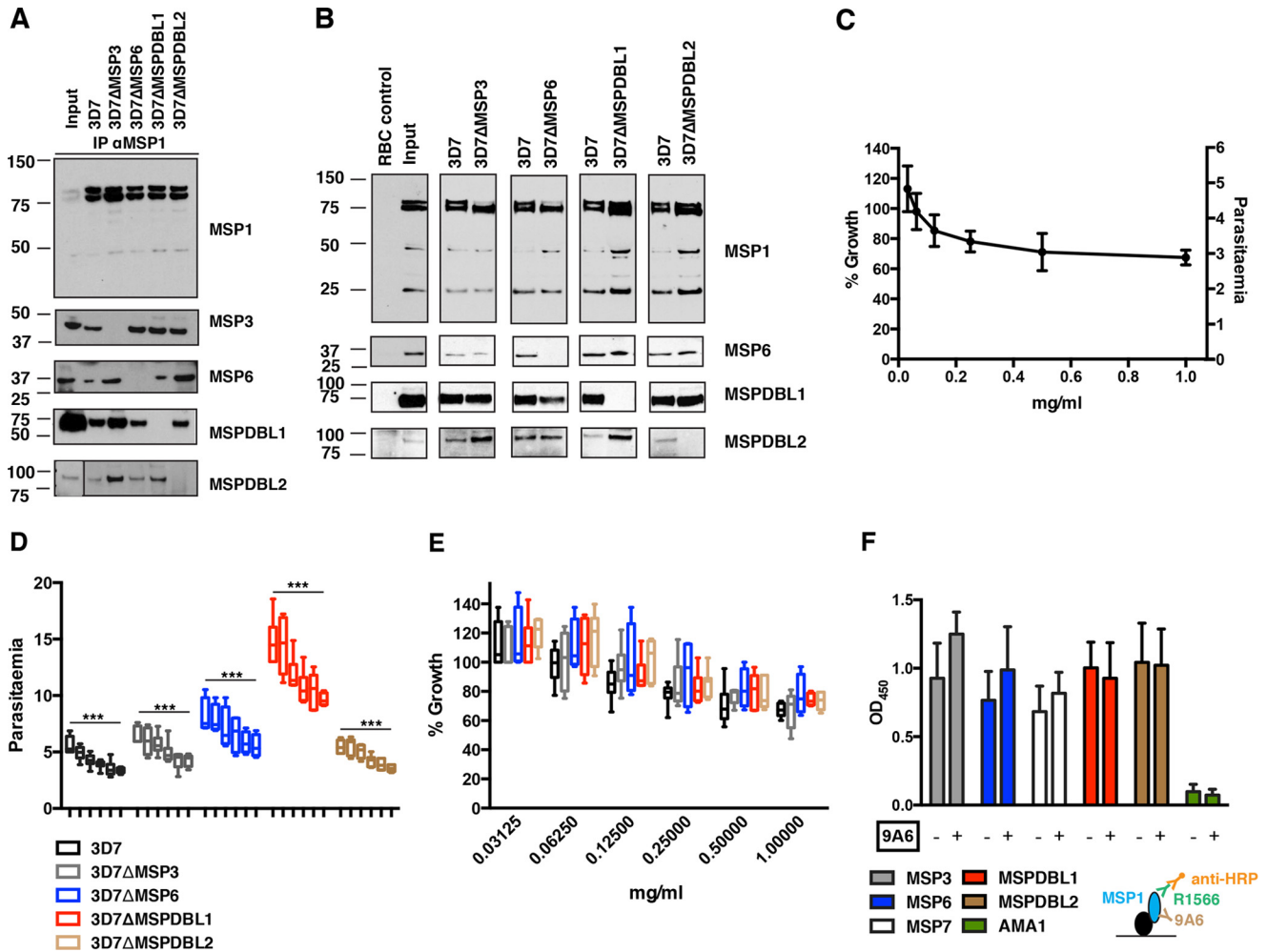


FIGURE 8. Single gene knockouts do not affect the erythrocyte-binding capacity of other MSP1 complexes. *A*, invasion supernatant of parasite lines 3D7, 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 were immunoprecipitated (IP) with anti-MSP1 p83 monoclonal antibody and probed with polyclonal R1566 (anti-MSP1), RMAL302 (anti-MSP3), R1467 (anti-MSP6), R1277 (anti-MSPDBL1), and R1296 (anti-MSPDBL2) antibodies. *B*, invasion supernatant from the 3D7, 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 parasite lines were incubated with human erythrocytes. Bound MSP1 complexes were detected on immunoblots using the polyclonal antibodies R1566 (anti-MSP1), R1467 (anti-MSP6), R1277 (anti-MSPDBL1), and R1296 (anti-MSPDBL2). *C*, growth assays using monoclonal antibodies for MSP1 p83 9A6 in a 2-fold increase in concentration from 0.03125 mg/ml to 1 mg/ml were used to determine the ability of these antibodies to inhibit parasite growth *in vitro*. In the presence of anti-MSP1 P83 antibodies at 1 mg/ml, growth was decreased by $40.46\% \pm 5.977\%$ ($p < 0.0001$). Error bars represent the mean \pm S.E. of five separate experiments in duplicate where significance was determined using Fisher's exact test. *D*, anti-MSP1 p83 antibodies were tested on 3D7, 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 in 2-fold increases in concentration from 0.03125–1 mg/ml, with the y axis showing changes in parasitemia levels over two cycles. In the presence of 1 mg/ml of anti-MSP1 p83 9A6, growth was decreased by $40.68\% \pm 7.004\%$ ($***, p = 0.0001$), $30.47\% \pm 8.183\%$ ($***, p = 0.0047$), $33.68\% \pm 5.382\%$ ($***, p < 0.0001$), and $34.28\% \pm 5.252\%$ ($***, p < 0.0001$) when 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 were compared with the 3D7 control, respectively. *E*, growth of the 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 parasite lines was compared with 3D7, and there was no significant growth difference between the 3D7ΔMSP3, 3D7ΔMSP6, 3D7ΔMSPDBL1, and 3D7ΔMSPDBL2 parasite lines in the presence of 1 mg/ml of anti-MSP1 p83 antibodies. Error bars represent the mean \pm S.E. of three separate experiments in duplicate where significance was determined using Fisher's exact test. For all growth inhibition studies, growth was measured as a percentage of the isotype control for the non-inhibitory MSPDBL2 7D11 monoclonal at the corresponding antibody concentration. *F*, the binding of MSP1 to each of the recombinant proteins (MSP3, MSP6, MSP7, MSPDBL1, and MSPDBL2) or the non-binder AMA1 was tested in the presence or absence anti-MSP1 p83. Error bars represent the mean \pm S.E. of three separate experiments in duplicate.

within the MSP1/6/7 complex. It has also been shown that blocking processing of MSP1 blocks egress, where unprocessed MSP1 is not able to bind to spectrin and is subsequently unable to destabilize the erythrocyte cytoskeleton for egress (46). It thus appears that the secondary processing of MSP1 is quite sensitive to interactions of the complex with antibodies during schizogony and also the egress of parasites for invasion, revealing at least two possible mechanisms for interference in parasite growth.

In this work we showed that there are multiple distinct MSP1 complexes on the parasite surface that bind to human erythrocytes. Previously, it has been shown that different MSPs on the

parasite surface bind to specific receptors on the erythrocyte, including evidence that MSP1 itself binds directly to receptors such as band 3 (5, 6, 47). The distinct MSP1 complexes that bind the erythrocyte contain MSP6, MSPDBL1, or MSPDBL2 (Fig. 9). MSPDBL1 and MSPDBL2 are ligands that have been shown to directly bind to the red blood cell surface, although the receptor(s) has/have not been identified. Interestingly, the MSP1 complexes containing these two receptor binding ligands vary significantly in size, with the MSP1-MSPDBL1 complex having a very large estimated molecular weight of more than 670 kDa. Although the MSP1-MSPDBL1 complex could be presented as an oligomer on the parasite surface, the

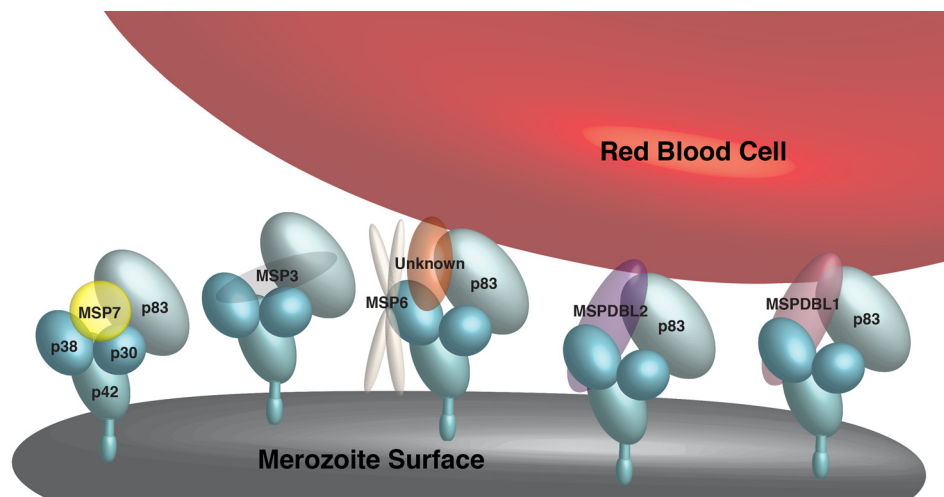


FIGURE 9. The peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 bind to MSP1 in independent complexes on the merozoite surface. The schematic shows different MSP1 complexes on the basis of our data and published reports. MSP1 anchors the peripheral MSPs MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 to form at least five MSP1 complexes that are presented on the merozoite surface. Of these, at least three complexes that contain MSP6, MSPDBL1, and MSPDBL2 mediate the interaction of the merozoite surface to receptors on the red blood cell. The MSP1-MSP6 complex binds to a red blood cell receptor either via the p83 fragment of MSP1 or through an unidentified MSP in the complex. We represented MSP3, MSPDBL1 and MSPDBL2 in the complex as a single unit for simplicity. However, it is likely that they are present as oligomers, as suggested by other work (16, 17, 56).

extremely large molecular weight suggests either the presence of other components present within this complex that have yet to be identified or a very elongated structure that elutes much earlier because of the inherent shape of this complex. In comparison, the MSP1-MSPDBL2 complex has an estimated molecular weight of ~ 150 kDa, suggesting a much smaller complex. MSPDBL1 and MSPDBL2 have very similar structural features and bind to the p42, p38, and p83 fragments of MSP1 and likely have overlapping functions (17, 19, 50). However, although they may perform a similar function overall, they are present in distinctly different MSP1 complexes, and the functional reason for this is not understood.

A third erythrocyte binding MSP1 complex we identified contains MSP6. However, this complex does not bind the host cell through MSP6, and it is possible that it binds erythrocytes via an unknown component or potentially through the p83 fragment, as suggested previously (Fig. 9) (6). However, it is interesting to note that there are other distinct MSP1 complexes that cannot bind erythrocytes and that these contain the MSP1 p83 fragment. For example, the MSP1-MSP3 and MSP1-MSP7 complexes both contain the MSP1 p83 fragment, but they are not able to bind directly to erythrocytes. It is possible that, in these MSP1 complexes, the p83 erythrocyte binding region is not accessible because of interactions with other MSPs. We did not observe any detectable binding of the native MSP1-MSP3 complexes to red blood cells despite a previous study reporting that synthetic peptides derived from MSP3 bind to erythrocytes (20). This discrepancy could be due to MSP3 peptide-binding epitopes being masked in the MSP1-MSP3 complex, and, therefore, they are not functionally relevant with respect to erythrocyte binding. Other potential roles of MSPs, except erythrocyte binding, have not been fully evaluated, although studies on MSP3 have described a novel protective role against heme release (51, 52). It is possible that other non-erythrocyte binding MSP1 complexes could play roles in evasion of host responses.

Functional redundancy of parasite ligands has been well known for *P. falciparum*, and it allows the parasite to evade host immune responses directed at blocking merozoite invasion (53, 54, 55). Genetic deletion of specific genes encoding MSP proteins had no effect on parasite growth or merozoite invasion, suggesting that these also show some functional redundancy. For example, when the erythrocyte-binding ligand MSPDBL1 was not present, both the MSP1-MSP6 and MSP1-MSPDBL2 complexes were still capable of binding to red blood cells. This suggests that various MSP1 complexes are able to compensate for each other. However, an antibody targeting a single epitope of the p83 fragment of MSP1 showed a reduction of overall parasite growth with modest antibody concentrations, as described previously. This antibody functions in a manner that is independent of blocking complex formation because the tested recombinant MSPs are still able to bind to MSP1 in the presence of 9A6. In addition, 9A6 can be used to immunoprecipitate native complexes, which suggests that this epitope is exposed within these complexes. Interestingly, naturally acquired p83 specific antibodies isolated from a semi-immune West African individual were also shown to efficiently inhibit parasite growth *in vitro* (21). Although more work has to be done to characterize the mode of action of this p83 monoclonal, this inhibition data presents p83 of MSP1 as a potential target, as its ability to inhibit growth is independent of the configurations of the various MSP1 complexes.

In summary, we identified distinct MSP1 complexes that vary in size, components, and function. MSP1 is utilized as an anchor for peripheral MSPs to interact with and be presented on the surface of merozoites. Importantly, although erythrocyte-binding MSP1 complexes are functionally redundant, targeting the main anchor of these complexes via the p83 fragment of MSP1 blocked parasite growth indiscriminative of any particular MSP1 complex. Further work to identify and understand how MSP1 complexes come together and function in invasion will greatly propel the ability to target

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functionally important MSP1 complexes to inhibit invasion of *P. falciparum*.

Author Contributions—C. S. L. conceived and designed the study, acquired the data, analyzed and interpreted the data, and drafted and revised the article. A. D. U. generated the knockout parasite lines, and revised the article. C. E. and H. B. purified MSP1 and revised the article. T. T. generated the MSP3 and MSP7 polyclonal antibodies and revised the article. P. E. C. and A. F. C. conceived and designed the study, analyzed and interpreted the data, and drafted and revised the article.

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