In Plasmodium falciparum malaria, erythrocyte invasion by circulating merozoites occurs via two distinct pathways involving either a sialic acid-dependent or -independent mechanism. Earlier, we identified two nonglycosylated exofacial regions of erythrocyte band 3 termed 5ABC and 6A as an important host receptor in the sialic acid-independent invasion pathway. 5ABC, a major segment of this receptor, interacts with the 42-kDa processing product of merozoite surface protein 1 (MSP142) through its 19-kDa C-terminal domain. Here, we show that two regions of merozoite surface protein 9 (MSP9), also known as acidic basic repeat antigen, interact directly with 5ABC during erythrocyte invasion by P. falciparum. Native MSP9 as well as recombinant polypeptides derived from two regions of MSP9 (MSP9/Δ1 and MSP9/Δ2) interacted with both 5ABC and intact erythrocytes. Soluble 5ABC added to the assay mixture drastically diminished the binding of MSP9 to erythrocytes. Recombinant MSP9/Δ1 and MSP9/Δ2 present in the culture medium blocked P. falciparum invasion into erythrocytes in vitro. Native MSP9 and MSP142, the two ligands binding to the 5ABC receptor, existed as a stable complex. Our results establish a novel concept wherein the merozoite exploits a specific complex of co-ligands on its surface to target a single erythrocyte receptor during invasion. This new paradigm poses a new challenge in the development of a vaccine for blood stage malaria.

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The abbreviations used are: EBA, erythrocyte-binding antigen; AD, Gal4 activation domain; BD, Gal4 DNA-binding domain; MSP, merozoite surface protein; RAP, rhoptry-associated protein; Trx, thioredoxin; Y2H, yeast two-hybrid; GST, glutathione S-transferase; pAb, polyclonal antibody; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.
3 termed 5ABC and 6A that function as a crucial erythrocyte receptor in the *P. falciparum* sialic acid-independent invasion pathway (16). 5ABC, a major segment of this receptor, interacted with MSP1α through its conserved 19-kDa C-terminal domain (MSP1α) and blocked the binding of MSP1α to intact erythrocytes (16). We also reported that the band 3 receptor interacts directly with a number of unidentified merozoite protein in incomplete Freund's adjuvant. The hybridoma supernatant was concentrated in water using Amicon Ultra-4/10K (Millipore). The 5ABC gene was subcloned into pET32a (Novagen) from pGBKTK7/5ABC using EcoRI and SalI restriction sites. Trx-5ABC was expressed in *E. coli* BL21(DE3) and affinity-purified using a His tag, and the color was stopped after 15 min by adding 2M H2SO4 (100 μl, the color) was measured at 450 nm. Residue low background binding as (A_	ext{non}} – 0.06 – 0.15) originating from the GST domain (GST control sample) was subtracted for each Trx-5ABC sample. The binding data were analyzed using SigmaPlot (Ligand macro for one-site saturation binding) assuming 1:1 binding between Trx-5ABC and the affinity-purified anti-His6 pAb. Background binding of Trx (containing His, tag) control samples to GST-MSP9/2, and GST was negligible (not shown) and was not subtracted. The dissociation constants (means ± S.E.) were determined from duplicate experiments.

Recombinant MSP9 Binding to Erythrocytes—In the binding assay, biotinylated GST-MSP9/2 (20 μM) or GST-MSP9/2 (20 μM) eluted from GST beads was incubated with human erythrocytes (50 μl, 50% hematocrit) in binding buffer A for 2 h at room temperature (final volume, 350 μl). The erythrocytes were sedimented (14,000 rpm, 30 s), washed twice with PBS, and lysed in 5.0 mM phosphate buffer (pH 8.0). The resulting erythrocyte ghosts were washed twice in PBS and subjected to SDS-PAGE followed by Western blotting using HRP-conjugated Neutravidin. Biotinylated GST was used as control. The binding inhibitory factor (MSP9/2 (30 μM) was incubated with biotinylated GST-MSP9/2 (20 μM) or GST-MSP9/2 (20 μM) in binding buffer B (2 h at room temperature; final volume, 300 μl). Human erythrocytes (50 μl, 50% hematocrit) were then added to this mixture and further incubated (2 h, room temperature). The erythrocytes were sedimented and analyzed as above by Western blot. Trx (30 μM) was used as control.

**Notice MSP9 and MSP1 Binding Assays—The** *P. falciparum* (3D7) culture supernatant (350 μl) prepared as described (16) was incubated with GST-MSP9/2 (20 μM), GST-MSP9/2 (20 μM), or GST-MSP9/2 (20 μM) conjugated to GST beads (50 μl, 50% slurry) at 4°C overnight. After low speed centrifugation, the beads were washed three times with PBS with 0.1% Tween 20. The proteins associated with the beads were analyzed by SDS-PAGE followed by Western blotting using rabbit anti-MSP9/2 pAb (for GST-MSP9/2 sample) or mAb 5.2 reactive against *P. falciparum* MSP1α (for GST-MSP9/2 and 2A samples). GST (20 μM) beads were used as control. Human erythrocytes (packed volume, 25 μl) were incubated with the *P. falciparum* culture supernatant (2 h at room temperature; 300 μl). As described above in the recombinant MSP9 binding assay, the erythrocytes were sedimented and analyzed by Western blot using anti-MSP9/2 pAb. Human erythrocytes incubated in only the culture medium were used as control. **Co-immunoprecipitation Assay—The** *P. falciparum* (3D7) culture supernatant (50 μl) and merozoite extract, prepared as described (16), were preincubated with protein G-agarose at 4°C for 24 h. The preclarified supernatant (400 μl) or protein extract (350 μl) was incubated with anti-MSP9/2 pAb (20 μl) at 4°C overnight, followed by protein G beads (40 μl, 50% slurry) at 4°C for 3 h. After centrifugation, the beads (pellet) were washed three times with PBS with 0.1% Tween 20. Immunoprecipitation was performed with the beads were analyzed by SDS-PAGE followed by Western blotting using mAb 5.2 and mAb 7H5/50 obtained from the Malaria Research and Reference Reagent Resource Center. The rabbit preimmune serum was used as control.

**RESULTS**

*P. falciparum* cDNA Library Screening—To identify malaria merozoite proteins interacting directly with the erythrocyte invasion receptor band 3, we carried out a Y2H screening of a *P. falciparum* (3D7 strain) cDNA library using the 5ABC segment (amino acids 720–761) of human band 3 as bait (see...
E. coli colonies were isolated from yeast cells and transformed into genes using the yeast mating method. Plasmids from positive \( P. falciparum \) (Fig. 1) of the frames. Two overlapping segments (MSP9/10 false positive library plasmids encoding incorrect reading analysis using the database at NCBI and PlasmoDB revealed plasmids were sequenced to identify the positive clones. Blast frames. Among the positive clones were several \( P. falciparum \) genes encoding hypothetical proteins.

Mapping MSP9-5ABC Interaction by Yeast Two-hybrid Assay—Based on the cDNA library screening results, we attempted to map the 5ABC-binding sites in the entire MSP9 polypeptide using the Y2H system. Three truncated MSP9 gene segments, namely MSP9/Δ3, MSP9/Δ4, and MSP9/Δ5 (Fig. 1), were cloned into pAD-GAL4-2.1 as a fusion to the Gal4 activation domain (AD). Recombinant pAD-GAL4-2.1 plasmids containing each of the three MSP9 truncations as well as MSP9/Δ1 and MSP9/Δ2 obtained from the library screen were transformed into yeast AH109 (Clontech). Recombinant pGBK7 plasmids containing segments of human band 3 gene corresponding to the 5ABC, 6AB (amino acids 807–842), and 3AB (amino acids 538–570) domains were prepared as a fusion to Gal4 DNA-binding domain (BD) and transformed into AH109 as described (16). The expression of MSP9 and band 3 constructs was confirmed by Western blotting using monoclonal antibodies (Santa Cruz Biotechnology) against the AD and BD (Fig. 2A). All of the fusion constructs except MSP9/Δ1 were stably expressed in yeast cells as soluble protein. Expression of AD-MSP9/Δ1 (calculated mass, 66.6 kDa) was observed as two lower molecular mass bands with sizable truncations predicted at the C terminus of the fusion protein (Fig. 2A, lane 2). Using recombinant pAD-GAL4-2.1 and pGBK7 plasmids, we performed Y2H assays by a co-transformation method in AH109 as described (16). AD-MSP9/Δ3, AD-MSP9/Δ4, and AD-MSP9/Δ5 did not interact with BD-3AB, BD-5ABC, and BD-6AB (Table I). On the other hand, both MSP9/Δ1 and MSP9/Δ2 interacted strongly with 5ABC but not with 6AB and 3AB under varying stringency conditions. These Y2H assay results confirm our cDNA library screening data obtained by the yeast mating method and suggest that there are two distinct 5ABC-binding domains located separately in MSP9/Δ2 and the N terminus of MSP9/Δ1.

Characterization of MSP9-Band 3 Interaction in Solution—To characterize the interaction of 5ABC with MSP9/Δ1 and MSP9/Δ2 identified in the Y2H system, we took an approach using solution binding methods. MSP9/Δ1 and MSP9/Δ2 were expressed in bacteria and purified as GST fusion proteins (Fig. 2B). Similar to expression in yeast, MSP9/Δ1 was expressed as two lower mass proteins (lanes 3 and 8) designated GST-MSP9/Δ1a (arrow; apparent mass, 45 kDa) and GST-MSP9/Δ1b (arrowhead; apparent mass, 29 kDa). These two GST fusion proteins had truncations at the C ter-
minus as shown by the Coomassie gel and anti-GST Western blotting analysis. Proteinolytic cleavage of the GST domain from the mixture of GST-MSP9/1a and GST-MSP9/Δ1b beads provided MSP9/1a (apparent mass, 17 kDa) in the supernatant (lane 2). MSP9/Δ1b could not be detected by SDS-PAGE, presumably because of its low molecular mass (<3 kDa). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of GST-MSP9/1a showed the exact mass of 46,420.3 Da (Fig. 2C). The N-terminal sequencing of this polypeptide showed that the entire GST domain (27.8 kDa) is intact (data not shown), indicating that the N and C termini of MSP9/1a are Lys-77 and Glu-235, respectively (Fig. 1). Similarly, the mass spectrometric analysis of GST-MSP9/Δ2 showed a mass of 29,827.7, indicating that the C terminus of MSP9/1b is Met-93. Expression and purification of GST-MSP9/Δ2 (apparent mass, 50 kDa; Fig. 2B, lanes 1 and 7) followed by a proteolytic cleavage of GST afforded soluble MSP9/Δ2 (apparent mass, 26 kDa; lane 2). Our attempt to express full-length MSP9 (lacking signaling peptide) as a GST fusion protein in bacteria was not successful as similarly noted by others (21).

To carry out a solution binding assay, we labeled GST-MSP9/1a (as a mixture of GST-MSP9/1a and GST-MSP9/Δ1b) and GST-MSP9/Δ2 with biotin and removed the GST domain by proteolysis. Biotinylated MSP9/1a and MSP9/Δ2 bound to GST-5ABC beads but not to control GST beads in the solution binding assay, demonstrating the direct and specific interaction of 5ABC with MSP9/1a and MSP9/Δ2 (Fig. 3A). Although same binding conditions were employed for both MSP9 constructs, different levels of biotin labeling between GST-MSP9/1a and GST-MSP9/Δ2 (not shown) precluded a further interpretation of the binding assay data. To analyze these interactions quantitatively, we carried out an ELISA using varying concentrations of soluble 5ABC expressed as a Trx fusion protein (Fig. 2D) and unlabeled GST-MSP9/Δ1a (as a mixture with GST-MSP9/1b) and GST-MSP9/Δ2 immobilized on the ELISA plate. Dissociation constants determined for the interaction of Trx-5ABC with MSP9/Δ1a and MSP9/Δ2 were 286 ± 53 nM and 945 ± 152 nM, respectively (Fig. 3B).

5ABC-mediated Binding of MSP9 to Erythrocytes—Biotinylated GST-MSP9/Δ1 (as a mixture of GST-MSP9/1a and GST-MSP9/1b) or biotinylated GST-MSP9/Δ2 was incubated with fresh human erythrocytes in suspension. GST-MSP9/Δ1a (Fig. 3C, lane 2, arrow) and GST-MSP9/Δ2 (lane 3, arrowhead) bound specifically to erythrocytes but not GST-MSP9/Δ1b (lane 2). Biotinylated GST (control) in an equivalent concentration did not interact with erythrocytes (lane 1), indicating that the interactions were specific to the MSP9/1a and MSP9/Δ2 domains. When soluble Trx-5ABC was added to the binding assay mixture, the erythrocyte interaction with both MSP9/1a (Fig. 3C, lane 4) and MSP9/Δ2 (lane 6) was drastically diminished. The Trx domain alone added to the assay mixture did not affect the binding (Fig. 3C, lanes 5 and 7). These results suggest that the binding of MSP9/1a and MSP9/Δ2 to erythrocytes is mediated by the 5ABC receptor. To obtain conclusive evidence supporting this implication, we produced a pAb against the MSP9/Δ2 domain in rabbit. The MSP9/Δ2 pAb reacted specifically to native MSP9 in the P. falciparum culture supernatant; lane 2, culture supernatant + GST-5ABC (20 µM) beads; lane 4, culture supernatant + GST (20 µM) beads; lane 5, culture supernatant + erythrocytes; lane 6, erythrocytes incubated in only the culture medium.

![Fig. 3. MSP9-erythrocyte interaction mediated by 5ABC. A. recombinant MSP9-5ABC interaction shown by pull-down assay in Western blot shows that biotinylated MSP9/Δ1a associated with GST-5ABC beads (lane 1) but not with GST control beads (lane 2). Similarly, biotinylated MSP9/Δ2 associated with GST-5ABC (lane 3) but not with GST (lane 4). For GST-5ABC and GST the final concentration was 20 µM. For biotinylated MSP9/Δ1 and MSP9/Δ2 the final concentration was 8 µM. B, the MSP9-5ABC interaction shown by ELISA. Concentration-dependent binding of soluble Trx-5ABC (0–12 µM) to GST-MSP9/Δ1a (top panel) and GST-MSP9/Δ2 (bottom panel) immobilized to a microplate is shown with background binding to the GST domain (control) subtracted from the data. The contribution of Trx domain to the binding interaction was insignificant (not shown). The dissociation constants (means ± S.E.) were estimated from duplicate experiments. The ligand binding curves are shown in the range of 0–6.5 µM Trx-5ABC. C, Western blot using HRP-conjugated Neutravidin shows that biotinylated MSP9/Δ2 bound to erythrocytes. Lane 1, erythrocyte + biotinylated GST (control); lane 2, erythrocyte + biotinylated GST-MSP9/Δ1; lane 3, erythrocyte + biotinylated GST-MSP9/Δ2; lane 4, erythrocyte + biotinylated GST-MSP9/Δ1 + Trx-5ABC; lane 5, erythrocyte + biotinylated GST-MSP9/Δ1 + Trx (control); lane 6, erythrocyte + biotinylated GST-MSP9/Δ2 + Trx-5ABC; lane 7, erythrocyte + biotinylated GST-MSP9/Δ2 + Trx (control). Arrow, GST-MSP9/Δ1; arrowhead, GST-MSP9/Δ2. For biotinylated GST-MSP9/Δ1, GST-MSP9/Δ2, and GST the final concentration was 20 µM. For Trx-5ABC and Trx the final concentration was 30 µM. D, Western blot analysis of rabbit anti-MSP9/Δ2 pAb (lanes 1 and 2) and native MSP9 binding to 5ABC and erythrocytes (lanes 3–5). Lane 1, anti-MSP9/Δ2 pAb reacted specifically to native MSP9 in the P. falciparum culture supernatant; lane 2, culture supernatant + GST-5ABC (20 µM) beads; lane 4, culture supernatant + GST (20 µM) beads; lane 5, culture supernatant + erythrocytes; lane 6, erythrocytes incubated in only the culture medium.

### TABLE I

**MSP9-band 3 interactions in yeast two-hybrid system**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>AD-MSP9</th>
<th>BD-band 3</th>
<th>Assay stringency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ1</td>
<td>3AB</td>
<td>–</td>
<td>SDV–T–L</td>
</tr>
<tr>
<td>Δ1</td>
<td>5ABC</td>
<td>–</td>
<td>SDV–T–L–H</td>
</tr>
<tr>
<td>Δ2</td>
<td>3AB</td>
<td>–</td>
<td>SDV–T–L–H–A</td>
</tr>
<tr>
<td>Δ3</td>
<td>3AB</td>
<td>–</td>
<td>SDV–T–L–H–A</td>
</tr>
<tr>
<td>Δ4</td>
<td>3AB</td>
<td>–</td>
<td>SDV–T–L–H–A</td>
</tr>
<tr>
<td>Δ5</td>
<td>3AB</td>
<td>–</td>
<td>SDV–T–L–H–A</td>
</tr>
<tr>
<td>Δ6</td>
<td>5ABC</td>
<td>–</td>
<td>SDV–T–L–H–A</td>
</tr>
</tbody>
</table>

*Note: SDV–T–L = strong direct and specific interaction.*
Fig. 4. Inhibition of *P. falciparum* reinvasion by soluble MSP9. Reinvasion inhibition assays were carried out using two independent *P. falciparum* (3D7) cultures with relatively low (1.8%) and high (6.5%) parasitemia. The assay results from both cultures were virtually identical as described in the text. The data obtained using the high parasitemia culture are shown. The percentage of parasitemia was calculated based on number of rings relative to the total erythrocytes counted (about 2,000 erythrocytes/sample) in Giemsa-stained smears. The assays were carried out in triplicates, and the data were plotted as the means ± S.D. Student’s *t* test was used to compare the mean.

ings demonstrate that MSP9 interacts directly with erythrocytes through its Δ1a and Δ2 domains utilizing the 5ABC region of band 3 as the receptor, presumably during *P. falciparum* merozoite invasion of erythrocytes.

Blocking of *P. falciparum* Reinvasion Using Recombinant MSP9—To investigate the biological significance of MSP9/Δ1a and MSP9/Δ2 domains binding to the host band 3 peptide 5ABC during erythrocyte invasion, we carried out an erythrocyte reinvasion inhibition assay in *P. falciparum* (3D7) culture as described (16) using soluble GST-MSP9/Δ1a (12 μM) and GST-MSP9/Δ2 (12 μM) as inhibitors. As compared with the GST (12 μM) control sample, both GST-MSP9/Δ1a and GST-MSP9/Δ2 samples showed drastically reduced reinvasion rates in cultures having relatively low (1.8%) as well as high (6.5%) parasitemia (Fig. 4). Relative reinvasion rates observed for each MSP9 construct were quite similar at both parasitemia levels; GST-MSP9/Δ1a samples showed 27% (*p* = 0.0002; 1.8% parasitemia) and 31% (*p* = 0.00006; 6.5% parasitemia) reinvasion rates, when the rate for the respective GST sample was taken as 100%. Reinvasion rates observed for GST-MSP9/Δ2 samples were 39% (*p* = 0.0002; 1.8% parasitemia) and 43% (*p* = 0.00002; 6.5% parasitemia). Inhibitor concentration at 2.4 μM showed a marginal effect and at 0.5 μM showed a statistically insignificant effect on reinvasion relative to the control sample (data not shown). We did not observe an accumulation of trophozoites and schizonts in any culture samples during the course of the reinvasion inhibition assay, indicating that the inhibitors added to the culture at the trophozoite stage did not affect intracellular maturation and release of the parasites. These results demonstrate that MSP9/Δ1a and MSP9/Δ2 specifically inhibit *P. falciparum* invasion into erythrocytes by binding to the 5ABC domain of band 3 on the uninfected erythrocyte surface. Our findings suggest that the MSP9-band 3 interaction as defined in this study plays a significant role in the erythrocyte invasion process.

MSP9 and MSP1 Forms a Co-ligand Complex—Previously, we showed that native MSP1Δ2 in the *P. falciparum* (3D7) culture supernatant directly associates with the 5ABC domain of band 3 (16). Because the results of this study indicate that native MSP9 in the same culture supernatant also interacts directly with 5ABC (Fig. 3D), we investigated the possibility of MSP9 and MSP1 existing as a complex of co-ligands binding the 5ABC receptor. In the solution binding assay, both GST-MSP9/Δ1a (as a mixture of GST-MSP9/Δ1a and GST-MSP9/Δ1b) and GST-MSP9/Δ2 beads pulled down native MSP1Δ2 from the

![Image](http://www.jbc.org/)

**Fig. 5.** MSP9-MSP1 complex. A, Western blot analysis of solution binding assay samples using mAb 5.2 reactive against MSP1Δ2 is shown. Lane 1, *P. falciparum* culture supernatant; lane 2, culture supernatant + GST-MSP9/Δ2 (20 μM) beads; lane 3, culture supernatant + GST (20 μM) beads; lane 4, culture supernatant only. B, Western blot using mAb 5.2 shows that native MSP1Δ2 in the culture supernatant co-immunoprecipitated with rabbit anti-MSP9/Δ2 pAb. Lane 1, culture supernatant + anti-MSP9/Δ2 pAb + protein G beads; lane 2, culture supernatant + rabbit preimmune sera + protein G beads; lane 3, culture supernatant only. C, Western blot using mAb 5.2 shows that MSP1Δ2 in the solubilized merozoite protein extract co-immunoprecipitated with rabbit anti-MSP9/Δ2 pAb. Lane 1, merozoite protein extract + anti-MSP9/Δ2 pAb + protein G beads; lane 2, merozoite protein extract + rabbit preimmune sera + protein G beads; lane 3, culture supernatant only. D, Western blot using mAb 7H8/50 shows that RAPI in the merozoite protein extract did not co-immunoprecipitate with rabbit anti-MSP9/Δ2 pAb. Lane 1, merozoite protein extract + anti-MSP9/Δ2 pAb + protein G beads; lane 2, merozoite protein extract + rabbit preimmune sera + protein G beads; lane 3, merozoite protein extract only; lane 4, culture supernatant only.

*P. falciparum* culture supernatant, whereas GST control beads did not (Fig. 5A). We then immunoprecipitated native MSP9 from the *P. falciparum* culture supernatant using rabbit anti-MSP9/Δ2 pAb. Western blotting of the immune complex using mouse mAb 5.2 against epitopes in the MSP1Δ2 domain (16) revealed that MSP1Δ2 co-immunoprecipitated with MSP9 (Fig. 5B, lane 1). Our anti-MSP9/Δ2 pAb does not cross-react with MSP1Δ2 or other parasite proteins based on Western blot analysis (Fig. 3D, lane 1). When rabbit preimmune serum was substituted for the pAb in the control immunoprecipitation sample, MSP1Δ2 was not detected by Western blot analysis (Fig. 5B, lane 2).

To rule out the possibility that the entire merozoite or its plasma membrane fragment contaminating the parasite culture supernatant could be co-immunoprecipitated, we used a solubilized parasite protein extract to repeat the above experiment. The protein extract was prepared from a mixture of parasite rhoptries (23), we were able to detect RAPI in the parasite protein extract (Fig. 5D, lane 3) but not in the culture supernatant (lane 4). In control samples using rabbit preimmune sera, MSP1Δ2 was not detected in the pellet containing protein G beads (Fig. 5, C and D, lanes 2). Together, our results demonstrate that native MSP9 and MSP1Δ2 form a specific complex as co-ligands binding the host invasion receptor 5ABC.
**DISCUSSION**

*P. falciparum* MSP9 is a highly conserved antigen that includes an unusual C-terminal acidic-basic tandem repeat region, an N-terminal hexapeptide tandem repeat region, and four cysteine residues in the N terminus (Fig. 1) (24). MSP9 is accumulated in the parasitophorous vacuole within intraerythrocytic parasites and at the surface of merozoites (19, 20, 24). Evidence suggests that trafficking MSP9 to the parasitophorous vacuole is linked to the localization of MSP3 to the vacuole space (25). MSP9 found in the culture medium upon schizont rupture becomes a part of immune complexes in the presence of immune serum (24). The function of MSP9 during erythrocyte invasion by merozoites has remained unknown. Although a chymotryptic-like protease activity has been reported for MSP9 (21, 26) with the implication of autoproteolysis (26), another putative natural substrate for MSP9 is yet to be identified. Interestingly, the serine protease catalytic triad postulated for *P. falciparum* MSP9 (His-55, Asp-94, and Ser-190 based on 3D7 sequence) is not found in *Plasmodium vivax*, *Plasmodium knowlesi*, and *Plasmodium cynomolgi* orthologues (27), arguing against the physiological relevance of the observed MSP9 protease activity in malaria parasite biogenesis.

Recently, Curtidor et al. (28) showed that 20-mer peptides representing certain regions of *P. falciparum* MSP9 bound to intact erythrocytes in a chymotrypsin-sensitive and a sialic acid-independent manner. Consistent with this finding, Kushwaha et al. (17) suggested that *P. falciparum* MSP9 interacts with erythrocyte band 3. The latter study, however, used a mixture of erythrocyte membrane proteins solubilized with a mild nonionic detergent as the source of band 3, which leaves open the possibility of indirect binding. It is now documented that even under ionic detergent-solubilizing (1% deoxycholate) conditions, band 3 maintains a macromolecular complex with its neighboring erythrocyte membrane proteins including the Rh complex (29). Nevertheless, Kushwaha et al. (17) reported that the putative band 3 interaction site lies in the N-terminal cysteine-rich region of MSP9 (amino acids 24–195). Our data obtained from the *P. falciparum* cDNA library screening, *in vitro* binding assay using the Y2H system (Table I), and independent solution binding assays (Fig. 3, A and B) unambiguously demonstrate a direct interaction between MSP9 and the 5ABC domain of band 3. Soluble 5ABC interacted with the direct binding of MSP9/11a and MSP9/12 to intact erythrocytes (Fig. 3C), implicating that the 5ABC domain of band 3 mediates the interaction of MSP9 with erythrocytes.

Mapping of the 5ABC-binding site in MSP9 showed there are N- and C-terminal sites represented by constructs MSP9/11a (amino acids 77–241) and MSP9/12 (amino acids 364–528), respectively. Because MSP9/3 (amino acids 184–363) did not interact with 5ABC, the N-terminal binding site could be located within amino acids 77–183. Neither the conserved four cysteines nor two tandem repeat regions participated directly in the MSP9-5ABC interaction. MSP9 did not interact with 3AB and 6AB constructs containing two other putative ectodomains of band 3 (Table I), underscoring the specificity of the interaction with 5ABC.

Dissociation constants we have estimated indicate that the interaction of 5ABC with MSP9/11a is somewhat stronger than with MSP9/12 (Fig. 3B). Consistent with the quantitative analysis of these interactions, MSP9/11a and MSP9/12 present in the culture medium at 12 μM blocked the *P. falciparum* reinvansion of erythrocytes by 69–73 and 57–61%, respectively, as compared with control samples (Fig. 4). It is possible that the two binding sites in MSP9 could form a single 5ABC-binding pocket in the native conformation. In any event, our data document for the first time that native MSP9 binds to intact erythrocytes as well as the 5ABC segment of band 3 (Fig. 3D). Collectively, our results demonstrate that MSP9 interacts directly with the erythrocyte surface during *P. falciparum* invasion, and the interaction is achieved through the 5ABC domain of band 3 and two specific regions of MSP9.

We reported earlier that the same 5ABC segment of band 3 interacts with *P. falciparum* MSP119 (16). Our co-immunoprecipitation experiments (Fig. 5, B and C) have unveiled a specific merozoite protein complex that includes MSP9 and MSP1.42. This co-ligand complex appears to play a direct role in binding merozoites to the host band 3 receptor during erythrocyte invasion. It is not clear, at present, whether MSP9 binds directly to MSP1.42, although native MSP1.42 in the culture supernatant associated specifically with recombinant MSP9/11a and MSP9/12 domains (Fig. 5A). MSP9 and perhaps MSP3 (25, 30, 31) could be a part of the previously identified merozoite protein complex containing MSP636 (32) and MSP722 (33, 34), believed to be attached to the merozoite surface through the interaction with glycosyl phosphatidylinositol-anchored MSP1.42.

Based on our findings here and in the earlier study (16), we propose two possible mechanisms for the merozoite-erythrocyte interaction in the band 3-dependent invasion pathway (Fig. 6). MSP1.42 and MSP9, the two band 3-binding co-ligands localized to the merozoite surface could bind to two different sections of a single 5ABC segment (Model I). Alternatively, only one of the two co-ligands might bind to a single 5ABC domain initially, but through equilibrium, each co-ligand in the co-ligand complex might interact at the same time with an independent 5ABC domain (Model II). In view of well established evidence that native band 3 exists as dimers and tetramers in the erythrocyte membrane (35–39), we favor Model II. It is plausible in the latter model that the merozoite makes an attachment to the erythrocyte surface through either MSP1-band 3 or MSP9-band 3 interaction and subsequently engages into a more stable host-parasite interaction by anchoring both co-ligands to two adjacent 5ABC domains in the band 3 tetramer or dimer. Furthermore, this anchoring process could be accompanied by some reorientation of the merozoite on the erythrocyte surface. On the other hand, it is conceivable that only one
of the co-ligands may interact with band 3 at a given time, and the existence of co-ligands is to simply provide redundancy in the merozoite-band 3 binding process.

The existence of a noncovalently associated complex of merozoite surface proteins including MSP6, MSP7, and processing products of MSP1 has been previously reported (12–34, 40). Moreover, published evidence indicates that soluble proteins in the parasitophorous vacuole such as MSP3 (25, 30), MSP9 (acidic basic repeat antigen) (19, 25), and S-antigen (41, 42) are also associated with the merozoite surface presumably by forming a direct or indirect noncovalent complex with a membrane-anchored merozoite surface protein. Our two proposed models (Fig. 6) introduce a novel concept in erythrocyte invasion by malaria parasites, wherein the merozoite exploits a specific noncovalent complex of co-ligands on its surface to target a single erythrocyte receptor. This new paradigm renews our understanding of the molecular mechanism by which malaria parasites invade erythrocytes and poses a new challenge in efforts to design and develop an effective vaccine for blood stage malaria.

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A Co-ligand Complex Anchors *Plasmodium falciparum* Merozoites to the Erythrocyte Invasion Receptor Band 3
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