Multiple pathways for *Plasmodium* ookinete invasion of the mosquito midgut

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***Plasmodium* ookinete invasion of the mosquito midgut is a crucial step of the parasite life cycle but little is known about the molecular mechanisms involved. Previously, a phage display peptide library screen identified SM1, a peptide that binds to the mosquito midgut epithelium and inhibits ookinete invasion. SM1 was characterized as a mimotope of an ookinete surface enolase and SM1 presumably competes with enolase, the presumed ligand, for binding to a putative midgut receptor. Here we identify a mosquito midgut receptor that binds both SM1 and ookinete surface enolase, termed "enolase-binding protein" (EBP). Moreover, we determined that *Plasmodium berghei* parasites are heterogeneous for midgut invasion, as some parasite clones are strongly inhibited by SM1 whereas others are not. The SM1-sensitive parasites required the mosquito EBP receptor for midgut invasion whereas the SM1-resistant parasites invaded the mosquito midgut independently of EBP. These experiments provide evidence that *Plasmodium* ookinetes can invade the mosquito midgut by alternate pathways. Furthermore, another peptide from the original phage display screen, midgut peptide 2 (MP2), strongly inhibited midgut invasion by *P. berghei* (SM1-sensitive and SM1-resistant) and *Plasmodium falciparum* ookinetes, suggesting that MP2 binds to a separate, universal receptor for midgut invasion.**

Malaria is currently the most devastating parasitic disease with an estimated death toll of over 1 million lives in 2010 (1). The life cycle of the malaria parasite requires invasion of five different cell types: Kupffer cells, hepatocytes, and erythrocytes in the human host (2–5) and midgut and salivary gland epithelial cells in the mosquito vector (6, 7). Of these, merozoite invasion of erythrocytes is the process studied in the most detail and the only one known to occur by multiple pathways (4).

Mosquito midgut invasion by *Plasmodium* ookinetes is currently considered a promising target for transmission-blocking intervention as parasite numbers undergo a major bottleneck at this stage (8, 9). After the mosquito ingests an infected blood meal, male and female gametes mate in the midgut lumen giving rise to zygotes that differentiate into motile ookinetes. After crossing the peritrophic matrix aided by chitinase secretion (10–12), the ookinete establishes specific molecular interactions with the midgut epithelial cells followed by their invasion and traversal. Several proteins from the ookinete (enolase, WARP, MAOP, PPLP5, SUB2, CelTOS, SOAP, P28, and P25) (7, 13–20) and the mosquito [aminopeptidase 1 (APN1), annexin-like proteins, carboxypeptidase B, croquemort scavenger receptor homolog, and calreticulin] (21–25) have been suggested to be involved in this process. However, the only molecular interaction between the ookinete and the midgut characterized thus far is the in vitro interaction between parasite Pvs25 and mosquito calreticulin (25).

Circumstantial evidence suggests that ookinete invasion of the mosquito midgut requires specific interactions between parasite and mosquito components (21, 26). In an attempt to elucidate these interactions at the molecular level, we have previously screened a phage display library for peptides that bind to the *Anopheles* midgut epithelium. This screen identified SM1, a dodecapeptide that binds to the midgut luminal surface and importantly, strongly inhibits *Plasmodium berghei* ookinete invasion (26). Midgut expression of the SM1 peptide by transgenic mosquitoes also inhibits *P. berghei* ookinete invasion (27). Further work indicated that SM1 structurally mimics the ookinete surface protein enolase, which we hypothesized to be involved in the recognition of a midgut receptor (7, 28).

Here we identify a mosquito midgut surface protein, enolase-binding protein (EBP), that binds both SM1 and ookinete surface enolase, and is required for midgut invasion. In addition, we provide evidence that *Plasmodium* ookinetes can invade the mosquito midgut by at least two alternate pathways, one sensitive and the other resistant to SM1 peptide inhibition. Finally, we identified a second peptide, midgut peptide 2 (MP2), that binds to a putative alternate receptor and inhibits ookinete midgut invasion of *P. berghei* (both SM1-sensitive and SM1-resistant) and *Plasmodium falciparum*. These findings have important

Significance

Malaria is among the most devastating parasitic diseases. Invasion of the mosquito midgut by motile malaria ookinetes requires specific interactions between proteins of both organisms. This study reports on a novel mosquito midgut receptor [enolase-binding protein (EBP)] that is recognized by an ookinete surface ligand (enolase). We also show that *Plasmodium* ookinetes invade the mosquito midgut by at least two different pathways: one dependent on and the other independent of the EBP–enolase interaction. Furthermore, we provide evidence for a second universal midgut receptor essential for midgut invasion by both human and rodent malaria parasites. These findings may lead to the development of novel targets for transmission-blocking interventions.

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implications for the development and implementation of malaria transmission-blocking strategies.

Results

Identification of the SM1 Receptor. Initial experiments characterized the binding properties of the SM1 peptide (Fig. 1*A*) to the mosquito midgut epithelium. To determine binding of SM1 to the midgut, *Anopheles gambiae* female and male midguts were dissected and opened into a sheet to expose the luminal side. SM1 binding was only detected on the surface of the female but not the male midgut (Fig. 1*B* and Fig. S1). Evidence that SM1 binds to the luminal (and not to the basal) surface of the midgut epithelium was previously reported (26). To further confirm binding of SM1 to the luminal side of the female mosquito midgut we incubated cross-sections of blood-fed *A. gambiae* female mosquito midguts with the SM1 peptide. Binding of SM1 was only detected along the luminal side of the midgut epithelium (Fig. 1*C*). To determine whether peptide binding occurs to a sugar moiety we chemically removed midgut surface carbohydrates using periodate treatment. This treatment had no effect on SM1 binding (Fig. 1*D*), suggesting that carbohydrates were not involved in the interaction. Next we tested whether SM1 structure plays a role in binding to its target. The SM1 dodecapeptide contains two cysteines at positions 2 and 11 that can make a disulfide bond thus giving rise to a loop of 8 aa (Fig. 1*A*). Linearization of the peptide either by reduction of the disulfide bond or by replacement of the two cysteine residues with alanine results in loss of the ability to bind to the midgut (Fig. 1*E*) indicating that the conformation of the peptide is important for binding.

The observation that SM1 binding to the mosquito midgut epithelium results in strong inhibition of *P. berghei* ookinete invasion raised the hypothesis that SM1 competes with an ookinete ligand for binding to a putative mosquito receptor. To identify the midgut protein(s) with which SM1 interacts we pulled down midgut proteins using a double-derivatized SM1 peptide carrying a biotin residue at its N terminus and a UV-activatable cross-linker attached to the 8-aa loop (Fig. S2). After incubation of the peptide with midgut sheets and UV irradiation, proteins cross-linked to SM1 were captured with streptavidin beads and then analyzed by SDS/PAGE. Four bands consistently present in experimental samples but not in controls were excised and analyzed by mass spectrometry (Fig. 2*A* and Dataset S1). This led to the identification of six candidate proteins, most of them midgut specific (Fig. 2*B*). To determine which proteins interact with SM1, we performed ELISAs by immobilizing each *A. gambiae* recombinant histidine-tagged protein on plastic wells and incubating with biotinylated SM1 peptide. These experiments revealed that only EBP is able to bind SM1 (Fig. 2*C*), suggesting that EBP may serve as receptor for SM1 and possibly an ookinete protein.

EBP is a single-copy gene that encodes a 407-aa protein (45.07 kDa) with no predicted glycosylation or myristoylation sites. It has a predicted 24-aa signal peptide at its N terminus and a predicted single-pass transmembrane domain at its C terminus (amino acids 367–384). EBP is a conserved gene in Culicidae mosquitoes, being 99.3% and 90.9% identical to its *Anopheles arabiensis* and *Anopheles stephensi* orthologs, respectively (Fig. S3). The degree of identity was lower for *Aedes aegypti* (53.1%) and *Culex quinquefasciatus* (31.2%). Immunofluorescence assays (IFAs) with an anti-EBP antibody (Fig. S3*C*) determined that the protein is located on the luminal surface of the mosquito midgut (Fig. 3*A*), which is consistent with the predicted secretion signal sequence, transmembrane domain, and its role as a putative receptor.

***Plasmodium* Enolase Interacts with Mosquito EBP.** Previous work showed that the anti-SM1 antibody recognizes *Plasmodium* enolase (7), implying that SM1 and a domain of the enolase protein share similar conformation. We hypothesized that enolase binds to EBP on the midgut surface via the domain resembling SM1. To test

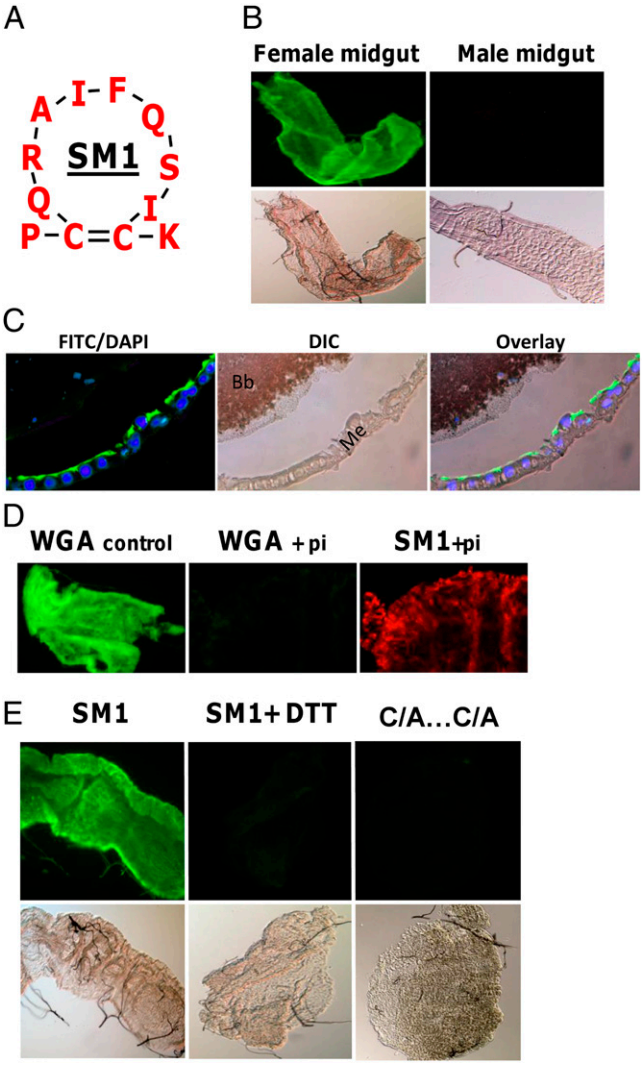

Fig. 1. SM1-midgut interactions. (*A*) SM1 structure. A disulfide bond between the two cysteines creates an 8-aa loop. (*B*) SM1 binds to female but not to male midguts. *A. gambiae* midgut sheets were incubated with a biotinylated SM1 peptide. SM1 binding was detected by incubation with FITC-labeled (green) streptavidin. Fluorescent images (*Upper*) and their corresponding light micrographs (*Lower*). (*C*) SM1 binds to the luminal side of the female *A. gambiae* midgut epithelium. Cross-sections of *A. gambiae* female mosquitoes after ingestion of a blood meal were incubated with biotinylated SM1 peptide. SM1 binding was detected by incubation with FITC-labeled (green) streptavidin. Bb, blood bolus; DIC, differential interference contrast microscopy; Me, midgut epithelium. (*D*) SM1 binding to the midgut is independent of protein glycosylation. (*Left*) Control midgut sheet incubated with FITC-labeled wheat germ agglutinin (WGA). (*Center*) Midgut sheet treated with periodic acid (pi) to remove sugar residues, incubated with FITC-labeled WGA. Periodic acid treatment abrogated WGA binding. (*Right*) Midgut sheet treated with periodic acid, incubated with biotinylated SM1 peptide followed by incubation with TexasRed-conjugated streptavidin. SM1 bound despite periodic acid treatment. (*E*) The disulfide bond is essential for midgut binding. (*Left*) Control biotinylated SM1 peptide. (*Center*) The biotinylated SM1 peptide was preincubated with DTT to disrupt the disulfide bond and methylated to yield a linear peptide. (*Right*) Mutant biotinylated SM1 peptide with the two cysteines replaced with alanines. Binding of biotinylated SM1 peptide followed by incubation with FITC-labeled streptavidin (*Upper*) and their corresponding light micrographs of the field (*Lower*).

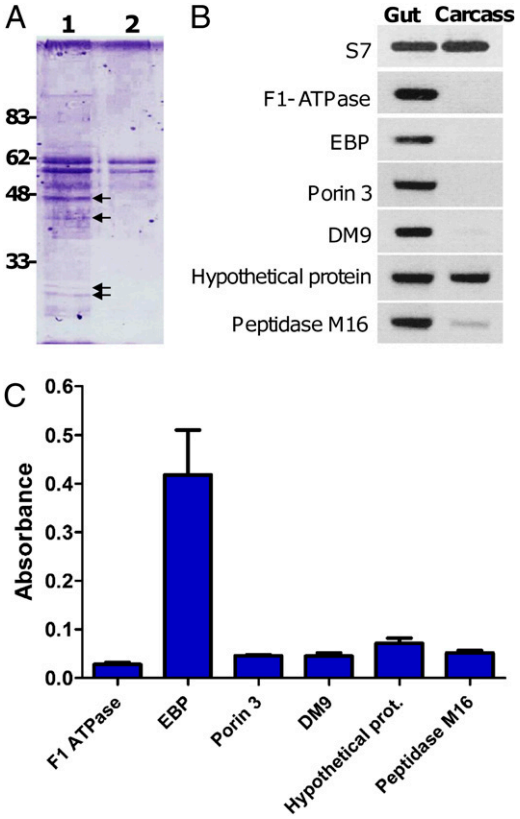

Fig. 2. Identification of SM1-interacting proteins. (*A*) Interacting proteins were analyzed using a pull-down approach (Fig. S1). A double-derivatized SM1 peptide carrying a biotin residue at its N terminus and a UV cross-linker attached to its loop was incubated with *A. gambiae* midgut sheets followed by UV irradiation to promote cross-linking to its target proteins. The peptide, with its cross-linked proteins, was captured on streptavidin beads followed by fractionation by SDS/PAGE and Coomassie Blue staining. Positions of size marker (in kilodaltons) migration are indicated on the left. Lane 1: complete procedure. Lane 2 (control): complete procedure, except that addition of the double-derivatized SM1 peptide was omitted. Arrows indicate bands consistently detected with the complete procedure but not in the control. (*B*) MS analysis of the four bands (arrows in panel *A*) identified the following six proteins: F1-ATPase (AGAP012081-PA), EBP (AGAP010479-PA), Porin 3 (AGAP009833-PA), DM9 (AGAP006398-PA), hypothetical protein (AGAP002756-PA), and peptidase M16 (AGAP000935-PA). The corresponding genes were analyzed by semiquantitative RT-PCR for expression in midgut and carcass (non-midgut) tissues. Ribosomal protein S7 served as a loading control. (*C*) Recombinant histidine-tagged proteins encoded by each candidate gene were immobilized on wells of a nickel-coated plate and incubated with biotinylated SM1 peptide. Peptide binding to each recombinant protein was detected by incubation with alkaline phosphatase-tagged streptavidin followed by incubation with a chromogenic substrate. Bars represent the mean absorbance from three independent experiments. Error bars represent the SEM.

this hypothesis, we incubated recombinant Pfenolase with midgut sections and found that enolase effectively binds to the luminal side of the midgut, where EBP is located (Fig. 3*B* and Fig. S4). This binding was outcompeted by excess SM1 peptide, indicating that binding was specific. We further investigated whether enolase can directly interact with EBP. Recombinant histidine-tagged AgEBP was immobilized on nickel–agarose beads followed by incubation with recombinant Pfenolase. Physical interaction of the two proteins could be detected and addition of SM1 peptide reduced binding of enolase to EBP (Fig. 3*C*). To quantify this interaction, recombinant AgEBP was immobilized on wells of a nickel-coated plate and incubated with recombinant Pfenolase in the presence of increasing concentrations of the SM1 peptide. The

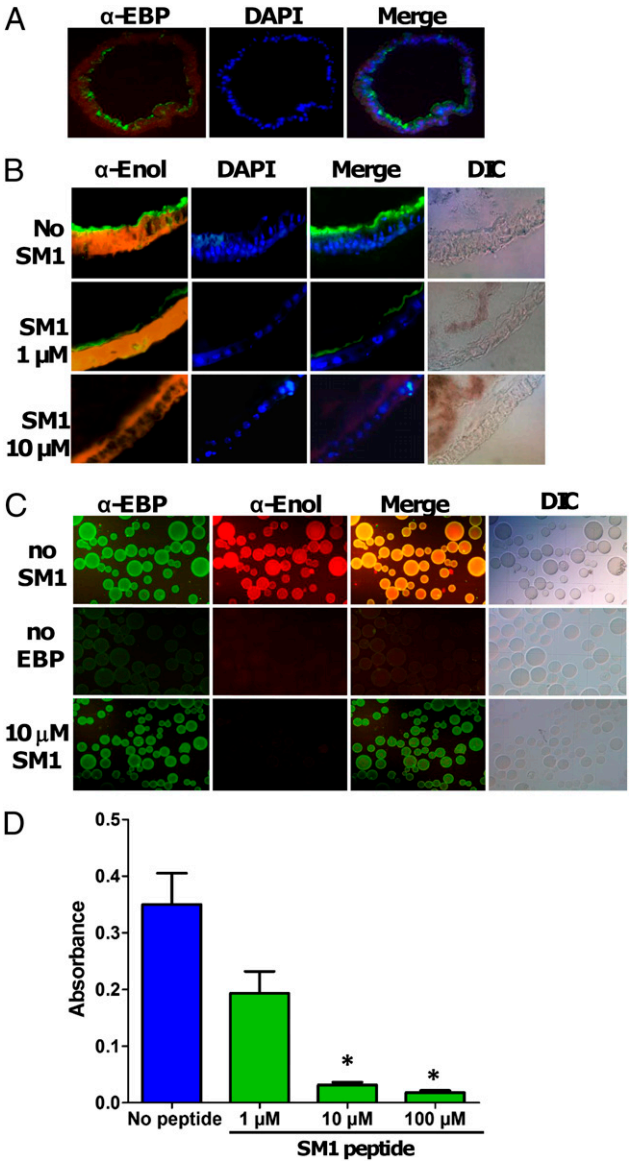

Fig. 3. EBP localization and interaction with parasite enolase. (*A*) IFA of an *A. gambiae* midgut cross-section probed with an anti-EBP antibody. The antibody detects EBP (green) on the luminal surface of the midgut. Nuclei are stained with DAPI (blue). (*B*) *A. gambiae* midgut sections were incubated with recombinant enolase and binding was detected with an anti-enolase antibody. Enolase binding was competed by addition of SM1 peptide. (*Top*) No peptide control. (*Middle* and *Bottom*) As indicated, 1 and 10 μM SM1. (*C*) Histidine-tagged recombinant EBP was immobilized on nickel–agarose beads. EBP was detected with an anti-EBP antibody (green) and binding of enolase was detected with an anti-enolase antibody (red). (*Top*) Binding of enolase to the immobilized EBP protein. (*Middle*) Control done as above but using beads that were not conjugated to recombinant EBP protein. (*Bottom*) Same experiment as in *Top* except that EBP beads were incubated with 10 μM SM1 peptide before the addition of recombinant enolase. SM1 inhibited the interaction of recombinant enolase with immobilized EBP. (*D*) His-tagged recombinant EBP was immobilized onto wells of a nickel-coated plate, incubated with the indicated concentrations of the SM1 peptide, followed by incubation with recombinant enolase. Binding of enolase to recombinant EBP was detected by incubation with an anti-enolase antibody and a secondary anti-rabbit IgG conjugated to alkaline phosphatase. Antibody binding was quantified by incubation with a chromogenic substrate. Bars represent the mean absorbance from three independent experiments. Error bars represent the SEM. Significance of differences with the no peptide control were determined by one-way ANOVA with Bonferroni's multiple comparison test (*$P < 0.0001$).

results indicate that recombinant Pfenolase physically interacts with recombinant AgEBP and that this interaction is specific as the SM1 peptide inhibited binding in a dose-dependent manner (Fig. 3*D*).

***P. berghei* Ookinetes Invade the Mosquito Midgut by More than One Pathway.** Transgenic mosquitoes that express and secrete SM1 into the midgut lumen inhibited *P. berghei* ookinete invasion by ~80% (27), even when a large excess of SM1 peptide was used. Incomplete blocking could result from an imperfect interaction between the SM1 peptide and EBP, or alternatively from a heterogeneous *P. berghei* population comprised of SM1-sensitive and SM1-resistant parasites. It is also possible that transgenic mosquitoes do not secrete enough SM1 or that the peptide might not be stable enough to mediate 100% inhibition. To test these hypotheses, *P. berghei* ANKA 2.34 parasites were sequentially passed through SM1-transgenic mosquitoes (Fig. 4*A*). If the original *P. berghei* population were composed of SM1-resistant and SM1-sensitive parasites, one would predict that the SM1-resistant parasites be preferentially selected by passage through the SM1 transgenic mosquitoes. This should not happen if partial inhibition were due to imperfect interaction of SM1 with its receptor, insufficient secretion of SM1 by the mosquito, and/or poor stability of the peptide. After the first passage throughout the transgenic mosquitoes, ~80% of the transmitted parasites became SM1 resistant. By the third passage, the parasite population became completely SM1 resistant (Fig. 4*B* and Dataset S2*A*). These results support the hypothesis that *Plasmodium* ookinetes invade the mosquito midgut epithelium by at least two pathways, one that is SM1 sensitive and another SM1 resistant. To confirm these results, the SM1-resistant parasite population obtained after the third passage was cloned. Two of the resulting clones were tested for their midgut invasion competence in wild-type and SM1 transgenic mosquitoes. Both clones were resistant to SM1 (Fig. 4*C* and Dataset S2*A*).

To obtain an independent estimate of the proportion of SM1-sensitive and SM1-resistant parasites in the original population, the ANKA 2.34 parasites were cloned by limiting dilution. Passive administration feeding assays (PAFAs) showed that midgut invasion by parasites from most of the clones were substantially inhibited (50–96%) by SM1 (Fig. 4*D* and Dataset S2*B*). This is in agreement with the previous results that the parental population was inhibited by ~80% (Fig. 4*B* and Dataset S2*A*). Two of the clones, R8 (~90% inhibition) and R9 (0% inhibition) displayed extreme phenotypes (Fig. 4*D* and Dataset S2*B*). Collectively, these results suggest that mosquito midgut invasion by *Plasmodium* ookinetes can occur via different pathways.

Ookinete Surface Enolase and Host Plasminogen Are Essential for Midgut Invasion by both SM1-Sensitive and SM1-Resistant Ookinetes. The current model for the SM1-sensitive invasion pathway proposes that recognition of the midgut epithelium is mediated by the interaction of ookinete surface enolase with mosquito EBP. In addition to EBP, enolase also binds plasminogen from ingested blood, an interaction required for midgut invasion (7). We investigated whether SM1-resistant and SM1-sensitive parasites required enolase, plasminogen, and EBP for midgut invasion. To this end we used two of the previously isolated *P. berghei* clones: R8 (the most SM1-sensitive clone) and R9 (a completely SM1-resistant clone) (Fig. 4*D* and Dataset S2*B*).

IFAs with an anti-Pfenolase antibody using nonpermeabilized R8 and R9 ookinetes showed that both display enolase on their surface at comparable levels (Fig. S5*A*). This result was confirmed by densitometric analysis of Western blots (Fig. S5 *D* and *E*). In addition, antibodies against the SM1 peptide (a mimotope of enolase) also immunoreacted with the surface of ookinetes from both clones (Fig. S5*B*). To analyze the requirement of surface enolase for ookinete midgut invasion we performed

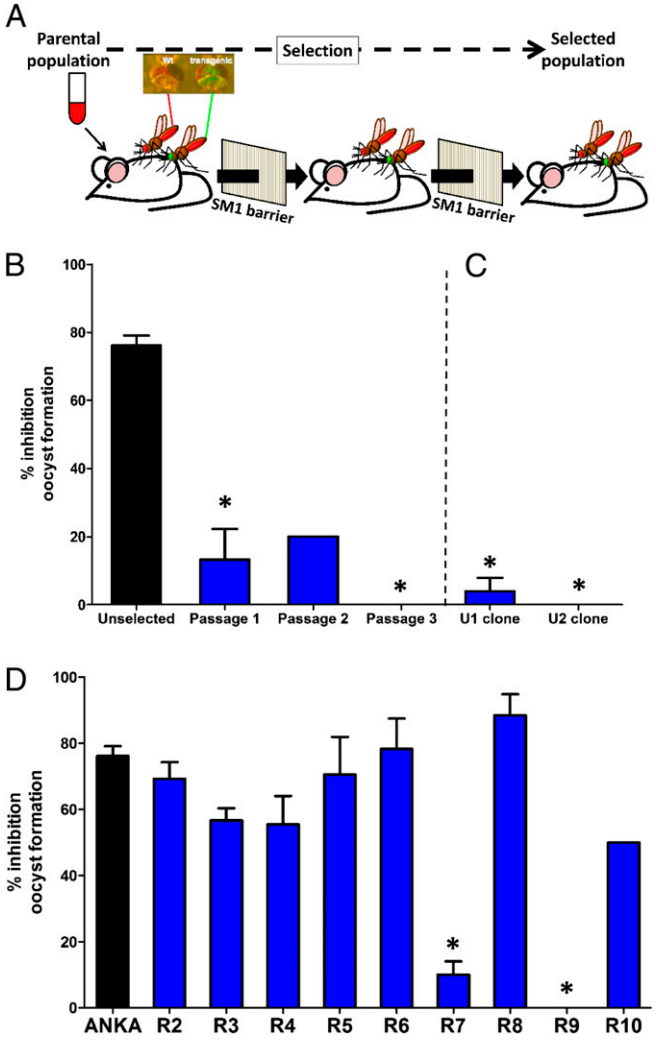

Fig. 4. Rapid selection of SM1-resistant *P. berghei*. (*A*) Selection started by feeding SM1-transgenic mosquitoes (green eyed) on a mouse infected with the parental, unselected *P. berghei* ANKA 2.34 population. To estimate inhibition of oocyst formation, wild-type mosquitoes (red eyed) that do not express SM1 were fed on the same mouse. Parasites that overcome the midgut SM1 barrier form SM1-resistant sporozoites that are used to infect another mouse. The selection process was repeated two more times. (*B*) Inhibition of oocyst formation after each passage. Note the rapid selection for resistant parasites. (*C*) *P. berghei* clones obtained from the parasite population after passage 3 are resistant to inhibition of midgut invasion by SM1-transgenic mosquitoes. (*D*) Individual *P. berghei* clones exhibit distinct SM1-inhibition phenotypes. Random clones obtained from the parental (unselected) ANKA 2.34 population were analyzed for SM1 inhibition either by experiments with transgenic and wild-type mosquitoes (similar to those illustrated in *A* and *B*) or by PAFAs using wild-type mosquitoes. For PAFAs, a group of mosquitoes (control) fed on a mouse infected with a given *P. berghei* clone. The mouse was then injected i.v. with 400 μg of the SM1 peptide and a second group of mosquitoes (experimental) fed on the same mouse. Oocyst numbers determined for the two groups of mosquitoes 12 d postinfection were compared with determine inhibition. Note that the majority of clones were sensitive to SM1. "ANKA" (*x* axis) represents unselected *P. berghei* ANKA 2.34 population. Bars represent the percent inhibition of oocyst formation from independent experiments shown in Dataset S2 *A* and *B*. Error bars represent the SEM. Percent inhibition of oocyst formation = [(control mean oocysts number − experimental mean oocysts number)/control mean oocysts number] × 100. Significance of differences with ANKA controls were determined by one-way ANOVA with Bonferroni's multiple comparison test (*$P < 0.0001$). Data from passage 2 in *B* was obtained from one experiment and was not included in the statistical analysis.

passive immunization feeding assays (PIFAs) with anti-Pfenolase antibodies. For both clones, oocyst formation was significantly inhibited by the antibody (Fig. 5*A* and Dataset S2*C*). These results suggest that surface enolase is required for successful midgut invasion by SM1-sensitive and SM1-resistant ookinetes.

IFAs with an anti-plasminogen antibody using nonpermeabilized ookinetes showed that host plasminogen is captured on the surface of both R8 and R9 ookinetes at comparable levels (Fig. S5*C*). To determine whether plasminogen is required for midgut invasion by each of the clones, PAFAs were performed with either aminocaproic acid (ACA; a lysine analog) or a 6-aa peptide encoding the enolase lysine motif (7). Lysine analogs, such as ACA, have been widely used to block the binding of the Kringle domains of plasminogen to specific lysine motifs present in plasminogen target proteins such as enolase. Both R8 (SM1-sensitive) and R9 (SM1-resistant) parasites had a significant reduction in oocyst numbers when fed to the mosquito in the presence of ACA (Fig. 5*B* and Dataset S2*D*). In a similar experiment, a peptide encoding the recently identified *Plasmodium* enolase lysine motif (the plasminogen-binding site on ookinete enolase) (7) was used to inhibit plasminogen binding to the surface of SM1-sensitive and SM1-resistant ookinetes. Administration of the enolase lysine motif peptide with the blood meal resulted in a significant reduction of oocyst numbers for both clones (Fig. 5*C* and Dataset S2*D*). These results suggest that SM1-sensitive and SMI-resistant ookinetes have a comparable requirement for host plasminogen during midgut invasion.

AgEBP Is Not Required for Midgut Invasion by SM1-Resistant *P. berghei* or by *P. falciparum* Ookinetes. EBP requirement for midgut invasion was examined by PIFAs with anti-AgEBP antibodies. PIFA with anti-AgEBP antibodies reduced oocyst formation in mosquitoes infected with the R8 SM1-sensitive clone by 68.3%, which is significantly different compared with the 14.2% inhibition of oocyst formation of R9 parasites (Fig. 5*D* and Dataset S2*E*). To examine the EBP requirement for midgut invasion by the human malaria parasite *P. falciparum* we performed standard membrane feeding assays (SMFAs) that incorporated anti-EBP antibodies. Anti-EBP antibodies did not significantly inhibit *P. falciparum* oocyst formation compared with controls (Fig. 5*E* and Dataset S2*F*).

To confirm these results, expression of mosquito EBP was knocked down by RNAi (Fig. S5*F*) followed by parasite feeding.

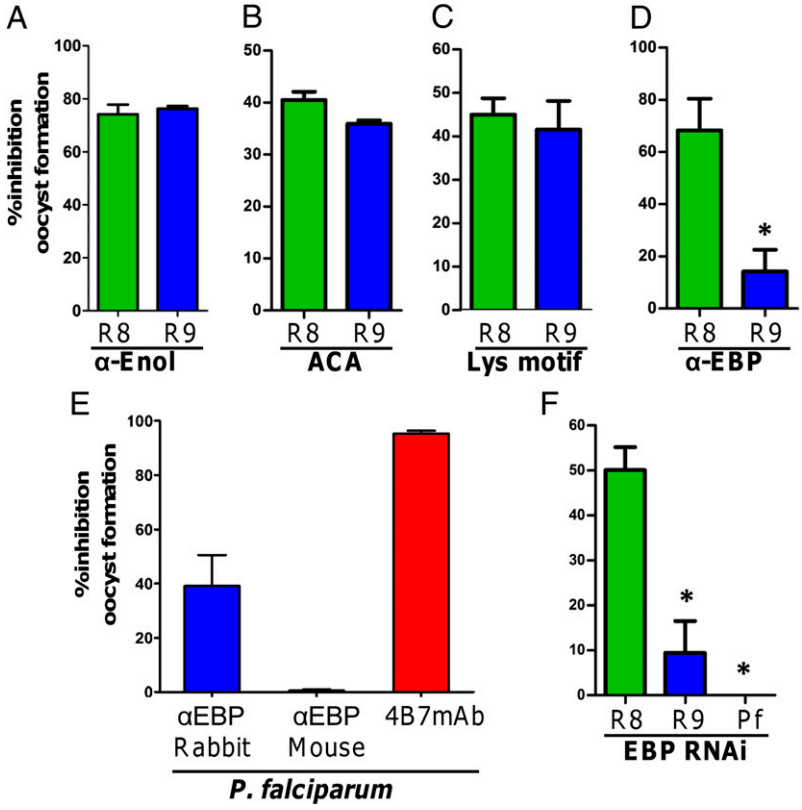

Fig. 5. Analysis of enolase, EBP, and plasminogen requirement for midgut invasion. (*A*) Requirement of parasite enolase for R8 and R9 midgut invasion was analyzed by PIFAs (similar to PAFAs in Fig. 4*D*) using rabbit anti-Pfenolase immune serum (1 mg per mouse). Anti-enolase antibodies inhibited midgut invasion of both R8 and R9 ookinetes. (*B* and *C*) The requirement of host plasminogen for midgut invasion by R8 and R9 ookinetes was analyzed by PAFAs (similar to Fig. 4*D*) with 400 μg per mouse of the lysine analog ACA (*B*) or the enolase lysine motif peptide (*C*). Oocyst formation by R8 and R9 parasites was similarly inhibited by ACA and the enolase lysine motif peptide suggesting that both clones require plasminogen for midgut invasion. (*D*) The requirement of EBP for midgut invasion of R8 and R9 ookinetes was tested by PIFAs with an anti-EBP antibody raised in mouse or rabbit. Significance of differences between the mean percent inhibition of pooled experiments of R9 compared with R8 (Dataset S2*E*) was determined by Student's *t* test (*$P < 0.05$). (*E*) The effect of EBP antibodies on midgut invasion of *P. falciparum* ookinetes was analyzed using SMFAs with anti-EBP antibodies produced in rabbit or mice. A monoclonal antibody (4B7) against the ookinete surface protein Pfs25 was used as positive control. (*F*) Effect of EBP knockdown on mosquito midgut invasion. Mosquitoes were injected with either EBP or GFP (control) double-stranded RNA. Four days postinjection, mosquitoes were fed on a mouse infected with either R8 or R9 parasites, or on a *P. falciparum* gametocyte culture. Inhibition of oocyst formation was determined by comparing oocyst numbers between the dsGFP- and the dsEBP-injected mosquitoes. Bars represent the percent inhibition of oocyst formation from independent experiments shown in Dataset S2 *E*–*G*). Percent inhibition of oocyst formation = [(control mean oocysts number − experimental mean oocysts number)/control mean oocysts number] × 100. Error bars represent the SEM. Significance of differences between the mean percent inhibition of pooled experiments of R9 and *P. falciparum* compared with R8 (Dataset S2 *E* and *F*) were determined by one-way ANOVA with Bonferroni's multiple comparison test (*$P < 0.05$).

Knockdown of AgEBP reduced oocyst numbers for R8 parasites by 50% which was significantly different compared with the 9.5% and 0% inhibition of oocyst formation for R9 and *P. falciparum* parasites, respectively (Fig. 5*F* and Dataset S2 *E* and *F*). These results indicate that *P. falciparum* and *P. berghei* SM1-resistant ookinetes do not require the EBP receptor for midgut invasion and suggest that these parasites invade the mosquito midgut via recognition of an alternate, yet unknown, receptor.

The MP2 Peptide Binds to a Putative Alternate Receptor for Ookinete Invasion. In a previous report, we described the identification of peptides with high binding affinity to the midgut luminal surface of *A. gambiae* female mosquitoes (26). SM1 was the most frequently recovered peptide (47.5% of the total) and a second peptide (ACYIKTLHPPCS), which we refer to as "MP2," was second in frequency (35% of the total). Similar to SM1, MP2 forms a disulfide bond between cysteines 2 and 11, resulting in the formation of an 8-aa loop (Fig. 6*A*).

To analyze whether the MP2 peptide interferes with midgut invasion by R8 SM1-sensitive and R9 SM1-resistant ookinetes we conducted PAFAs with synthetic peptide and transmission-blocking experiments with transgenic bacteria engineered to secrete the SM1 or the MP2 peptides in the lumen of the mosquito midgut (Fig. S6) (29). SM1 only inhibited oocyst formation of the R8 and R6 clones (Fig. 6*B*) as reported in Fig. 4. The MP2 peptide significantly inhibited midgut invasion of both R8 SM1-sensitive (71.4% inhibition) and R9 SM1-resistant parasites (52.4% inhibition) (Fig. 6*B* and Dataset S2 *H* and *I*). In addition, we tested sensitivity to MP2 peptide for two additional *P. berghei* clones (R6 SM1 sensitive and R7 SM1 resistant) obtained from the parental ANKA 2.34 (Fig. 4*D*). Midgut invasion of both additional clones (R6, 88.7%; R7, 67.0% inhibition) was significantly inhibited by the MP2 peptide (Fig. 6*B* and Dataset S2 *H* and *I*).

To analyze the effect of the MP2 peptide on midgut invasion by *P. falciparum* ookinetes, SMFAs were performed in the presence of the SM1 or MP2 synthetic peptides, or by transmission-blocking experiments with SM1- or MP2-secreting bacteria. Midgut invasion by *P. falciparum* ookinetes was not significantly inhibited by SM1 (Fig. 6*C* and Dataset S2*J*). In contrast, a significant reduction in oocyst numbers (71.3% inhibition) was detected when MP2 was incorporated into the infectious blood meal, compared with control mosquitoes. Moreover, there was no significant inhibition when mosquitoes were fed with the MP2–C11A peptide (Fig. 6*C* and Dataset S2*J*). The MP2–C11A peptide has a substitution of alanine for cysteine at position 11 which prevents disulfide bond and loop formation but is otherwise identical to MP2 (Fig. 6*A*). These results support the hypothesis that the MP2 peptide binds to a universal mosquito midgut receptor required for midgut invasion of ookinetes from different *Plasmodium* species.

Discussion

Several lines of evidence support the hypothesis that SM1 binds to a surface protein on the luminal side of the mosquito midgut epithelium that mediates *Plasmodium* ookinete invasion (26). Pull-down experiments with the double-derivatized SM1 peptide identified six different potentially interacting proteins. However, of the six recombinant proteins, only EBP interacted strongly with the SM1 peptide, establishing this protein as a prime SM1 receptor candidate. Midgut-specific expression and protein localization on the luminal surface of the midgut is consistent with its function as a receptor. EBP is a novel protein, well conserved among *Anopheles* mosquitoes, and with no homology to any protein domain previously described. Other midgut proteins located on the midgut luminal surface, including APN1, annexin-like proteins, and calreticulin, have been investigated as potential receptors for ookinete invasion (21, 22, 25). APN1 is currently considered a target for a transmission-blocking vaccine, as anti-APN1 antibodies strongly inhibit midgut invasion by *Plasmodium* ookinetes (21, 30). However, the mechanism by which APN1 supports midgut invasion of *Plasmodium* ookinetes is still unknown as no ookinete interacting protein has been identified. *Anopheles* annexin-like proteins have also been shown to be important for invasion of *Plasmodium* ookinetes and it has been suggested that the ookinete might use annexins for protection or to facilitate traversal of the invaded cell (22). Of the above-mentioned mosquito receptor candidates, calreticulin is the only

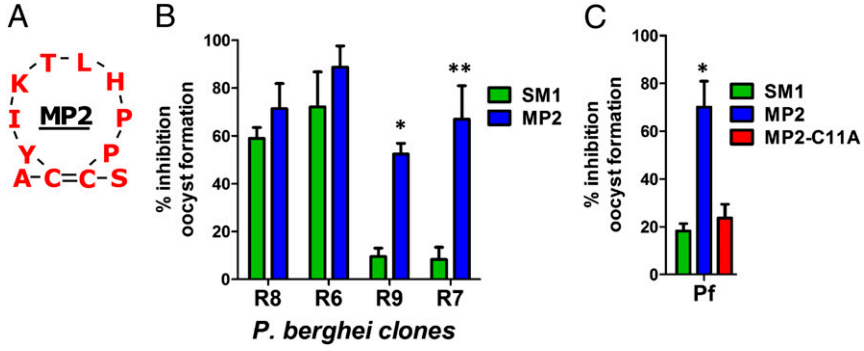

Fig. 6. The MP2 peptide inhibits midgut invasion by *P. berghei* and *P. falciparum* ookinetes. (*A*) Diagrammatic representation of the MP2 peptide. Note the disulfide bond between cysteines 2 and 11 resulting in the formation of an 8-aa loop. (*B* and *C*) Peptide inhibition experiments were performed with either synthetic peptide or with *P. agglomerans* bacteria engineered to express the SM1 or the MP2 peptide (Fig. S6) (29). (*B*) For *P. berghei*, PAFAs were performed by injecting mice with either the SM1 or MP2 peptides (400 μg per mouse as described in Fig. 4). Percent inhibition was determined by comparing the number of oocysts per midgut before and after peptide injection. Significance of the differences of SM1 and MP2 inhibition were determined by Student's *t* test (*$P <$ 0.001, **$P < 0.05$). (*C*) For *P. falciparum*, either 400 μg/mL synthetic peptide or 1× PBS/5% DMSO (control) were added to *P. falciparum* gametocyte cultures and fed to *A. gambiae* mosquitoes using SMFAs. Percent inhibition was determined by comparing the oocyst number per midgut between control and peptide treatment at day 8 postinfection. For the experiments with peptide-expressing bacteria, mosquitoes were fed on wild-type or engineered bacteria suspended in a sugar solution and 1 d later, fed on a mouse infected with one of the *P. berghei* R clones or fed on a *P. falciparum* gametocyte culture. Percent inhibition was determined by comparing the oocyst number per midgut between mosquitoes fed with wild-type bacteria and those fed with transgenic bacteria. MP2 inhibited ookinete midgut invasion of all parasites tested. Bars represent the percent inhibition of oocyst formation from data pooled from independent experiments with synthetic peptide and engineered bacteria as shown in Dataset S2 *H–J*. Percent inhibition of oocyst formation = [(control mean oocysts number − experimental mean oocysts number)/control mean oocysts number] × 100. Error bars represent the SEM. Significance of SM1 and MP2 inhibition were determined by one-way ANOVA with Bonferroni's multiple comparison test (*$P < 0.05$).

one shown to interact with a specific parasite protein, Pvs25 (25). However, no functional studies have been performed to determine the significance of this interaction for ookinete midgut invasion.

Previously we reported that the SM1 peptide is a mimotope of ookinete surface enolase, as anti-SM1 antibodies recognize this protein (7). Because SM1 binds to EBP on the midgut lumen, we hypothesized that ookinete enolase also interacts with mosquito EBP and in this way mediates midgut invasion. The results reported here provide further support for this model. First, recombinant enolase binds to the epithelial cell surface. Second, enolase binding to the epithelial cell surface is outcompeted by the SM1 peptide. Finally, recombinant EBP directly interacts with recombinant enolase and this binding is competitively disrupted by the SM1 peptide. From these observations we infer that ookinete surface enolase binds to mosquito EBP on the luminal midgut surface and that this interaction is required for midgut invasion by certain *Plasmodium* parasites. Several pathogens, including bacteria, fungi, and protozoans, use nonconventionally secreted proteins such as enolase as adhesins to recognize and bind to the target tissue they invade (31). However, only a few enolase-interacting proteins from the targeted tissue have been identified thus far, such as the extracellular matrix protein fibronectin (22–34) and human colon (cyto)keratin-8 (35).

Our data suggest that mosquito EBP is not required for midgut invasion by *P. falciparum* and by SM1-resistant *P. berghei* parasites, indicating that these parasites invade the midgut by recognizing an alternate receptor. Surprisingly, antibodies against parasite enolase inhibited midgut invasion of all parasites tested: SM1-sensitive and SMI-resistant ookinetes (this work) and of *P. falciparum* ookinetes (7). Based on the differential SM1 sensitivity, it was expected that R9 and *P. falciparum* ookinetes would be insensitive to anti-enolase antibodies as they use a receptor different from EBP. This expectation was supported by our observation that the predicted amino acid sequence of R8, R9, and the published ANKA 2.34 enolase gene (www.plasmodb.org) are identical and by the comparable enolase expression levels between R8 and R9 parasites. Given that ookinete surface enolase is likely to have dual functions—binding to the EBP receptor and capturing plasminogen from the host serum (7)—we hypothesize that anti-enolase antibodies inhibit invasion by interfering with the binding of plasminogen to ookinete surface enolase. Alternatively, anti-enolase antibodies could inhibit interaction of the ookinete with the midgut epithelium by steric hindrance.

To date, the only *Plasmodium* invasion process shown to take place by alternate pathways is the merozoite invasion of red blood cells (RBCs) (4, 5). Merozoites can invade the RBC by sialic acid-dependent or acid-independent pathways using multiple merozoite ligands [e.g., erythrocyte binding-like (EBL) and *P. falciparum* reticulocyte binding-like proteins, PfRh] and multiple RBC receptors (glycophorins, complement receptor 1, basigin, and unknown receptors). Importantly, *P. falciparum* merozoites are able to switch from the sialic acid-dependent to the sialic acid-independent pathway when neuramidase-sensitive parasites are cultured for several cycles with neuramidase-treated erythrocytes (36). Similarly, we selected and isolated *P. berghei* parasite clones

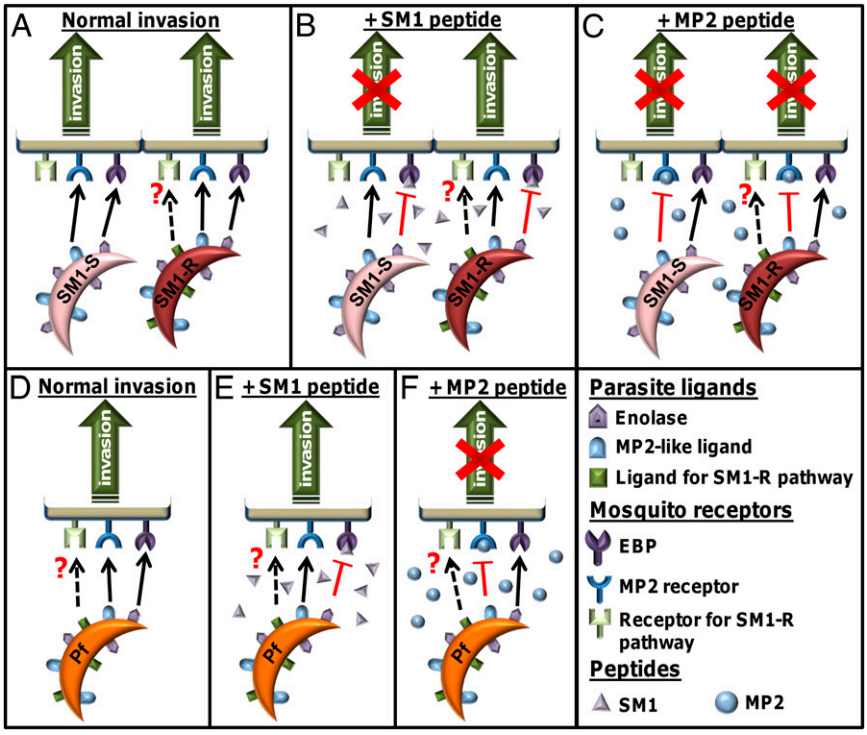

Fig. 7. Model for multiple steps and alternate pathways of ookinete midgut invasion. (*A*–*C*) *P. berghei* ookinetes. Keys to the identity of the molecules are given (*Lower Right*). (*A*) SM1-sensitive (SM1-S) ookinetes require the interaction of (*i*) parasite surface enolase with mosquito surface EBP and (*ii*) parasite MP2-like ligand with mosquito MP2 receptor for successful invasion of the midgut. These interactions may occur concomitantly or sequentially. The two interactions may also occur for SM1-resistant (SM1-R) ookinetes. (*B*) In the presence of excess SM1 peptide, the interaction between parasite enolase and mosquito EBP is disrupted, inhibiting invasion by SM1-S ookinetes. SM1-R parasites either bypass the enolase–EBP interaction step or potentially recognize a third mosquito receptor (the receptor for SM1-R pathway). (*C*) In the presence of excess MP2 peptide, invasion of the mosquito midgut is inhibited for both SM1-S and SM1-R ookinetes. The MP2 peptide blocks an essential interaction between a putative ookinete MP2-like ligand and a putative mosquito MP2 receptor. (*D*–*F*) *P. falciparum* ookinetes behave as SM1-R *P. berghei* and can invade the mosquito midgut in the presence of excess SM1 peptide but not in the presence of excess MP2 peptide. Given that the MP2 peptide inhibits both *P. falciparum* and *P. berghei* invasion, this step may be universally required for midgut invasion by any *Plasmodium* species.

that are resistant to the inhibitory effect of the SM1 peptide during mosquito midgut invasion. Our results show that *Plasmodium* ookinetes invade the mosquito midgut epithelium by at least two independent pathways: SM1 sensitive and SM1 resistant. Selection of SM1-resistant parasites from the parental ANKA 2.34 line resulted in parasites fully resistant to SM1 after only three passages. The speed of selection suggests that the resistant parasites may have already been present in the parental ANKA 2.34 and did not involve a switch as reported for merozoite invasion of RBCs (36). The SM1-resistant and SMI-sensitive phenotypes of independent clones obtained from the unselected parental stock lend support to this hypothesis.

The independent phenotypes of the different plasmodia vis-à-vis SM1 and MP2 peptide inhibition suggest that midgut invasion is a multistep process similar to the merozoite invasion of the RBC. As for the MP2 pathway, the interaction of merozoite PfRh5 with RBC basigin is a step required for RBC invasion by all of the *P. falciparum* strains tested so far (37). Similar to the SM1 pathway, invasion of RBCs is still maintained after disruption of individual EBL genes (EBA-175, EBA-181, and EBA-140) and PfRh (PfRh1, PfRh2a, PfRh2b, and PfRh4) (4). We propose that ookinete invasion of the mosquito midgut is a multistep process that requires the interaction of multiple parasite ligands with multiple mosquito receptors.

There are about 40 species of *Anopheles* mosquitoes worldwide that can transmit the five species of *Plasmodium* that infect humans. These parasites must have evolved to develop in its corresponding *Anopheles* species. Moreover, it is becoming increasingly evident that field strains of *Plasmodium* can vary in terms of their ability to infect different malaria vectors (38–41). Conceivably, this variability is due in part to variations in the ability of each parasite to recognize and invade the midgut of a given *Anopheles* species. As for RBC invasion (4), the ability to invade the mosquito midgut by multiple pathways is conceivably advantageous to the parasite, as it might allow it to infect different mosquito species displaying variant midgut receptors.

In summary, we have identified EBP as a putative mosquito midgut receptor and characterized its interaction with an ookinete surface enolase. This interaction is competitively inhibited by the SM1 peptide and is essential for midgut invasion by certain *Plasmodium* strains. Moreover, we report that *Plasmodium* ookinetes are able to invade the mosquito midgut by at least two pathways, both of which are inhibited by the MP2 peptide. We envision ookinete midgut invasion as a multistep process involving the interaction between multiple parasite ligands and mosquito receptors (Fig. 7).

Materials and Methods

Ethics Statement. This project was carried out in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (42). The animal protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (Protocol M009H58). Anonymous human blood used for parasite cultures and mosquito feeding was obtained under institutional review board (IRB) Protocol NA 00019050 approved by the Johns Hopkins School of Public Health Ethics Committee. The IRB waived the need for written informed consent from the participants (blood donors).

Peptide Pull-Down Assays and MS Analysis. Midguts were dissected and kept on ice in the presence of protease inhibitors before thorough washing with several changes of PBS to remove cell debris and other contaminating materials. Pull-down of the midgut proteins and the liquid chromatography–tandem MS analysis were as described (6).

Measurement of EBP–Enolase Interaction. EBP–enolase interaction was measured by immobilizing recombinant EBP to agarose beads or wells in a 96-well plate and incubating with recombinant enolase. Specificity of the interaction was determined by adding increasing concentrations of SM1 before the addition of recombinant enolase. Details are provided in *SI Materials and Methods*.

Selection of SM1-Resistant Clones. Selection of SM1-resistant parasites was done by sequentially passing the parental ANKA 2.34 parasites through SM1-transgenic *A. stephensi* mosquitoes (27). Details are provided in *SI Materials and Methods*.

In a separate set of experiments, we isolated clones from the parental the unselected ANKA 2.34 population using the same limiting dilution approach. These clones were tested for sensitivity to SM1 inhibition by one of the two alternate procedures described next. The concentration of SM1 peptide secreted into the midgut of transgenic mosquitoes is unknown. We also used two alternate procedures to deliver SM1 peptide to the mosquito midgut lumen: (*i*) injecting SM1 peptide i.v. into infected mice before mosquito feeding as described by Ghosh et al. (26) and (*ii*) administering to mosquitoes recombinant bacteria that express the peptide before providing an infectious blood meal, as described under *Transmission-Blocking Assays with Transgenic Bacteria*.

***P. berghei* PIFA or PAFA.** The PIFA and PAFA procedures are the same, except that for PIFA an antibody is injected and for PAFA a peptide or another small molecule is injected i.v. into the mouse. In each case, *A. gambiae* mosquitoes are fed on a *P. berghei*-infected mouse before (control) and after (experimental) injection of the experimental molecule. Then the number of oocysts per mosquito is compared between the two groups to determine the transmission-blocking efficiency. Details are provided in *SI Materials and Methods*.

***P. falciparum* SMFA.** *P. falciparum* gametocyte cultures were diluted to 0.1% gametocytemia and fed to *A. gambiae* and *A. stephensi* mosquitoes using glass membrane feeders. Details are provided in *SI Materials and Methods*.

Transmission-Blocking Assays with Transgenic Bacteria. Transmission-blocking experiments with transgenic *Pantoea agglomerans* engineered to secrete SM1 or MP2 peptides were performed as previously described (29). Further details are provided in *SI Materials and Methods*.

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