

MAEBL Is Essential for Malarial Sporozoite Infection of the Mosquito Salivary Gland

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Abstract

Malarial sporozoites mature in the oocysts formed in the mosquito midgut wall and then selectively invade the salivary glands, where they wait to be transmitted to the vertebrate host via mosquito bite. Invasion into the salivary gland has been thought to be mediated by specific ligand–receptor interactions, but the molecules involved in these interactions remain unknown. MAEBL is a single transmembrane-like protein that is structurally related to merozoite adhesive proteins. We found MAEBL of the rodent malaria parasite, *Plasmodium berghei*, to be specifically produced by the sporozoites in the oocyst and localized in their micronemes, which are secretory organelles involved in malarial parasite invasion into the host cell. A targeted disruption experiment of the *P. berghei* MAEBL gene revealed that it was essential for sporozoite infection of the salivary gland and was involved in the attachment to the salivary gland surface. In contrast, the disruption of the MAEBL gene did not affect sporozoite motility in vitro nor infectivity to the vertebrate host. These results suggest that *P. berghei* MAEBL is a sporozoite attachment protein that participates in specific binding to and infection of the mosquito salivary gland.

Key words: parasitology • malaria • disease transmission • insect vectors • gene targeting

Introduction

Malaria remains one of the most devastating infectious diseases in the world. The development of effective strategies to limit its transmission is one of the most important objectives of malaria control programs. Therefore, an understanding of the biology of the malarial parasite in the mosquito vector is required. Transmission of the malarial parasite occurs when sporozoites are injected from the salivary gland into the host skin by bites from an infected mosquito. These malarial sporozoites are generated in oocysts formed on the mosquito midgut and released into the body cavity of the mosquito by oocyst rupture. They then selectively invade the salivary gland. There, they become infective to the vertebrate host and wait for the mosquito to bite (1–3). Because sporozoite infection of the mosquito salivary glands is a key step for malarial parasite transmission to the vertebrate host, efforts have been made to elucidate this mechanism. Some experiments suggest that sporozoite invasion into the salivary gland is mediated by specific ligand–receptor interactions (2–5). For instance, it was reported that antiserum against the salivary gland and lectins that recognize the salivary gland surface carbohydrates in-

hibited sporozoite invasion into the gland (5). However, the molecules mediating such specific interactions remain to be identified.

MAEBL genes were first identified in the rodent malaria parasites *Plasmodium yoelii* and *Plasmodium berghei* (Pb),* and seem to be highly conserved throughout the *Plasmodium* species including *Plasmodium falciparum*, a medically important human malarial parasite (6). MAEBL is an ~200-kD protein with a single transmembrane-like domain and is structurally related to members of the *Plasmodium* Duffy binding-like (DBL) family (7–8), which includes the Duffy antigen binding proteins of *Plasmodium knowlesi* and *Plasmodium vivax* (9), and the 175K erythrocyte binding antigen of *P. falciparum* (10). These proteins are produced in the merozoite stage, localized in micronemes, and believed to recognize host-specific erythrocyte surface receptors and participate in a junction formation necessary for merozoite entry into the host erythrocyte (11). MAEBL was reportedly produced in the merozoite stage (6) and based on structural similarities is classified as a member of a recently proposed expanded merozoite adhesive protein family, named the *ebf* family, which includes DBL family proteins and putative erythrocyte binding proteins encoded by

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*Abbreviations used in this paper: DBL, Duffy binding-like; Pb, *Plasmodium berghei*; TRAP, thrombospondin-related adhesive protein.

newly identified *P. falciparum* genes, such as *ebl-1*, *baebl*, *pebl*, *jesebl*, and *maebl* (8). Among these members, MAEBL is exceptional in that its ligand binding domains are similar to those of apical membrane antigen 1 (12) rather than those of the DBL domain, as it was reported to be localized not in the microneme but in the rhoptry (13). Recently, Kappe et al. (14) reported that *P. yoelii* MAEBL is also produced in the sporozoite stage, which suggests its involvement in sporozoite infection.

In this paper, we report about the specific production of MAEBL by the sporozoites in the oocyst and its essential role in sporozoite infection of the salivary gland. The structural relation to the merozoite adhesive proteins, the subcellular localization, and the phenotype resulting from MAEBL gene disruption strongly suggests that MAEBL plays an essential role in the sporozoite as an attachment protein that is especially committed to the invasion of the salivary gland.

Materials and Methods

Parasite Preparations. BALB/c mice infected with Pb ANKA strain were prepared by peritoneal injection of infected blood that was stored at -70°C . Infected mice were used within one blood passage for mosquito biting. For the purification of sporozoites, infected mosquitoes were anesthetized in CO_2 20–24 d after the infective blood meal. Hemolymph was collected from a pinhole in the abdomen by the injection of 10 μl medium 199. The salivary glands and midgut were then dissected out, washed in saline, and separately collected in 70 μl medium 199 on ice. Collected tissues were gently ground in the medium to release sporozoites. After the removal of tissue fragments by centrifugation at 18 g for 3 min, sporozoites were collected from the supernatant by centrifugation at 5,000 g for 3 min.

For the purification of merozoites, infected rat blood was cultured for 16 h in medium RPMI 1640 (GIBCO BRL) containing 20% fetal calf serum, under 10% O_2 , and 5% CO_2 . Mature schizonts were purified from the cultured blood by density gradient using Nycoprep 1.077A (AXIS-SHIELD). For Western blot analysis, merozoites were additionally purified by erythrocyte lysis.

Assays for Sporozoite Gliding Motility and Infectivity to Rats. Gliding motility and infectivity of sporozoites were assessed as described by Sultan et al. (15). For gliding motility assay, sporozoites were collected from the hemolymph of mosquitoes 24 d after an infective blood meal and kept for 2 h at 4°C in medium 199 containing 3% BSA. Gliding on an uncoated microscope slide was then observed under a phase-contrast microscope. For infectivity assay, sporozoites in the oocysts were collected from the mosquito midgut 24 d after an infective blood meal and intravenously injected into 3-wk-old Wistar rats. Their parasitemia was checked daily by a Giemsa-stained blood smear, and infection rates and the prepatent period of infection were determined.

Antibody Preparation and Western Blot Analysis. For antibody preparation, the peptides ERKKAEEAAKKAEEERKRIE and KLIGGRWFKEVAFDEIVEDYDKVYTLAMISNEQI, which correspond to the amino acid sequences of the repeat region and the COOH-terminal region of PbMAEBL, respectively, were synthesized. Each peptide was conjugated to keyhole limpet hemocyanin as a carrier and used for the immunization of rabbits. Specific antibodies were affinity purified from the antisera using a

normal human serum-activated High Trap column (Amersham Pharmacia Biotech) linked with the respective peptides.

Western blot analysis was performed as previously described (16). In brief, purified parasites were homogenized in SDS-PAGE sample buffer containing 1% SDS and 5% 2-ME and boiled for 5 min. Parasite proteins (10^6 sporozoites or 3×10^6 merozoites per lane) were separated by SDS-PAGE on a 8% gel and electrophoretically transferred to a nitrocellulose membrane. The blotted membrane was blocked in PBS containing 5% skimmed milk, incubated for 60 min with primary antibodies diluted in the same buffer (20 $\mu\text{g}/\text{ml}$ final concentration), washed, and then incubated for 60 min with alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad Laboratories) diluted at 1:1,000 in the same buffer. After washing, signals were obtained using the AP Conjugate Substrate Kit (Bio-Rad Laboratories).

Immunofluorescence Microscopy. Immunofluorescence microscopy was performed as previously described (16). In brief, purified parasites on glass slides were fixed in acetone for 2 min and rinsed in PBS. The slides were then blocked in PBS containing 1% BSA and incubated for 60 min with primary antibodies diluted in the same buffer (20 $\mu\text{g}/\text{ml}$ final concentration). After being rinsed five times in PBS, the slides were incubated for 60 min with FITC-conjugated anti-rabbit IgGs (Zymed Laboratories), diluted at 1:40 in the same buffer, and then rinsed an additional five times in PBS. For nuclear staining, DAPI (0.02 $\mu\text{g}/\text{ml}$ final concentration) was added to the secondary antibody solution. Samples were mounted in PermaFluor (Thermo Savant) and micrographs were obtained with an Olympus BX60 fluorescence microscope with a C4742-95 digital color camera (Hamamatsu Photonic System). The images were processed using AquaCosmos (Hamamatsu Photonic System) and Adobe Photoshop.

Immunolectron Microscopy. Purified parasites were fixed in 0.1 M phosphate buffer (pH 7.4) containing TAAB (1% paraformaldehyde, 0.1% glutaraldehyde) for 15 min on ice. They were dehydrated in ethanol and embedded in LR gold resin (London Resin Company Ltd.). Ultrathin sections were picked up on 200-mesh nickel grids, blocked for 30 min in PBS containing 0.01% Tween 20 and 5% nonfat dry milk (blocking buffer), incubated at 4°C overnight with primary antibodies diluted in blocking buffer (20 $\mu\text{g}/\text{ml}$ final concentration), washed three times with PBS containing 0.01% Tween and 10% Block Ace (Dainippon Laboratories), incubated for 1 h with goat anti-rabbit IgG conjugated to gold particles (15-nm diameter; AuroProbe, Amersham Pharmacia Biotech) diluted at 1:40 in blocking buffer, and washed six times. Finally, the sections were fixed with 2.5% glutaraldehyde for 10 min, rinsed twice in distilled water, and stained with 2% uranyl acetate and Reynold's lead citrate. These samples were examined by a HITACHI H-800 transmission electron microscopy at an accelerating voltage of 100 kV.

Targeted Disruption of the PbMAEBL Gene and Thrombospondin-related Adhesive Protein Gene (TRAP). The targeting vector for the PbMAEBL gene was constructed as follows. Two partial DNA fragments of the PbMAEBL gene were amplified by PCR using genomic DNA as a template with these primer pairs: 5'-GGCGAGCTCTTATTATTTGCTTCATGTTGTATT-3' and 5'-GCCGGATCCTTTATCCAAGTTCATCGAAAATGT-3'; and 5'-GGCCTCGAGGATGAATATAATGAAGATTTCTTA-3' and 5'-GCCGGTACCAGATGAAAAACATCTACCATT-ATT-3'. These fragments were subcloned into either side of the selectable marker gene in pBluescript using the unique restriction sites, SacI/BamHI and XhoI/KpnI, respectively. The targeting vector for the PbTRAP gene was constructed in the same manner but using these two primer pairs: 5'-GGCGAGCTCGTA-

CATCTTGTGCTTGAAGC-3' and 5'-GCCGGATCCTGACCATTAAGAAACACAC-3'; and 5'-GGCCTCGAGAAGAAGTATGTAACGAACAAATCG-3' and 5'-GCCGGTACCGCAACTTTTTCTACTTCCTGAC-3'. For the gene targeting experiment, the plasmid was completely digested with the restriction enzymes *SacI* and *KpnI* to release the linear targeting construct. The gene targeting experiment was performed as previously described (17).

Southern Blot Analysis. Southern blot analysis was performed as previously described (16). In brief, *Pb* genomic DNA extracted from blood stage parasites (2 μ g) was completely digested with a restriction enzyme, *EcoRV* or *EcoT22i*. The fragments were separated on 1.2% agarose gel and transferred to nylon membrane. Partial digestion fragments of *PbMAEBL* and *PbTRAP* genes were amplified by PCR using genomic DNA as the template with the primer pairs, 5'-CTAAACAACCATGTAAACATAAAATGG-3' and 5'-ATCCAAGTTCATCGAAAATGTTTTTCC-3', and 5'-TTATTGGATGGCTCAGGAAGTATT-3' and 5'-CTGGTTCTGCTGGTTCTATAGGTT-3', respectively. The products, labeled with [³²P]dCTP, were used as hybridization probes.

Results

MAEBL Is a Microneme Protein Produced by the Sporozoite in the Oocyst. In the course of an unpublished expressed sequence tag study of the *Pb* sporozoites, we found that expressed sequence tags of *PbMAEBL* transcripts were abundantly included. To confirm MAEBL protein production in this stage, we performed immunofluorescence microscopy, immunoelectron microscopy, and Western blot analysis. Through these experiments we used two distinct antibodies raised against synthetic peptides corresponding to the repetitive region and the cytoplasmic region of *PbMAEBL*, respectively. In addition, we performed the same experiments in the intraerythrocytic stage parasites because MAEBL was reported to be a merozoite protein (6). In immunofluorescence microscopy, strong staining was observed in the cytoplasm of mature sporozoites collected from the oocyst (midgut) and mainly in the anterior half, which is a typical pattern of microneme staining (Fig. 1 A, left). In contrast, staining was greatly reduced in sporozoites from the salivary gland (Fig. 1 A, middle). Merozoites were not stained by either antibody (Fig. 1 A, right). In immunoelectron microscopy, *PbMAEBL* was detected in the micronemes of mature sporozoites in the oocyst (Fig. 1 B, left). On the other hand, neither antibody showed any specific affinity against the apical organelles of merozoites, including rhoptries where MAEBL has been reported to be localized (Fig. 1 B, right) (18). By Western blot analysis, *PbMAEBL* was detected as an \sim 200-kD protein in oocyst sporozoite preparations but not in the merozoite stage (Fig. 1 C). These results demonstrate that *PbMAEBL* is a microneme protein specifically produced by sporozoites in the oocyst, which indicates a possibility that *PbMAEBL* participates in sporozoite infection of the salivary gland.

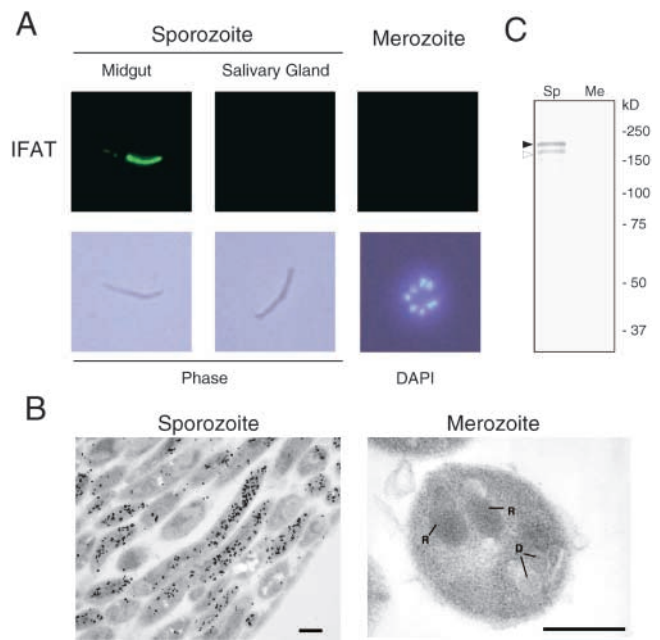


Figure 1. Stage-specific production of *PbMAEBL* and localization in the microneme. (A) *PbMAEBL* is specifically produced by sporozoites in the oocyst. Immunofluorescent staining was performed in sporozoites collected from the midgut (left), sporozoites collected from the salivary glands (middle), and schizonts (right). Parasites were fixed with acetone for 2 min, incubated with polyclonal rabbit antibodies against a synthetic peptide corresponding to the amino acid sequence of the repeat region of *PbMAEBL*, and then conjugated with FITC with second antibodies. Oocyst sporozoites were stained mainly in the anterior half, which is a typical pattern for microneme staining. The corresponding phase-contrast image or DAPI staining images are shown below. (B) *PbMAEBL* is localized in micronemes of oocyst sporozoites. Immunoelectron microscopy was performed in the infected mosquito midgut (left) and cultured mature merozoites (right). The midgut was dissected 18 d after an infective blood meal. Merozoites were purified from infected blood after culture for 16 h *in vitro*. They were fixed and embedded in LR Gold resin. Ultrathin sections were incubated with the same antibodies as used in A and then conjugated with gold particles (15 nm) with second antibodies. Antibodies were attached to the micronemes of oocyst sporozoites but not to the rhoptries (R) and showed no specific affinities to merozoites. D, dense body; bars, 0.5 μ m. (C) *PbMAEBL* was detected as an \sim 200-kD protein only in the sporozoites. Western blot analysis of *PbMAEBL* was performed in lysates of sporozoites in the oocysts (Sp) and merozoites (Me). Sporozoites (10^6) and merozoites (3×10^6) were lysed in the SDS-PAGE sample buffer containing 0.5% SDS, separated on a 8% acrylamide gel, and analyzed by immunoblotting with the same antibodies as in (A). *PbMAEBL* was detected as a major \sim 200-kD protein band (closed arrowhead) with a minor band of its putative degraded product (open arrowhead).

MAEBL Is Essential for Sporozoite Infection of the Salivary Gland. To investigate the function of *PbMAEBL*, we generated *PbMAEBL* gene-disrupted parasites by homologous recombination (Fig. 2, A and C) (17). Recombinant populations, *maebl*(-) 1 and 2, were separated from wild-type populations, *maebl*(+) 1 and 2, by limiting dilution. To exclude the possibility that all of the *maebl*(-) populations were derived from a single clone, two other *maebl*(-) populations, *maebl*(-) 3 and 4, were prepared independently. We also generated *PbTRAP* gene-disrupted parasite populations, *trap*(-) 1 and 2, which are known to have no infectivity to either the salivary gland or rat liver cells

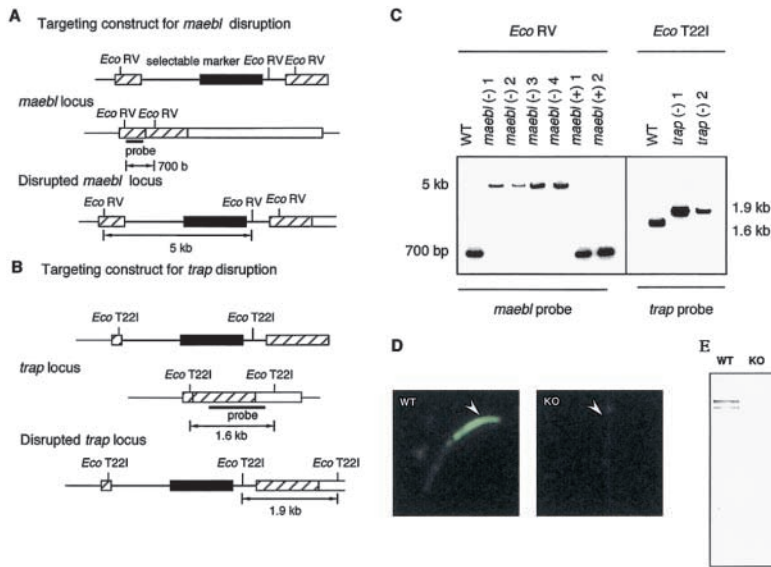


Figure 2. Targeted disruption of the PbMAEBL and PbTRAP gene. (A) Schematic representation of the targeted disruption procedure of the PbMAEBL gene. The targeting vector (top) containing a selectable marker gene is integrated into the PbMAEBL gene locus (middle) by double crossover. This recombination event results in the disruption of the PbMAEBL gene locus (bottom) and confers pyrimethamine resistance to PbMAEBL gene-disrupted parasites. The probe used for Southern blot analysis (C, left) is indicated by a solid bar. The recognition sites for EcoRV and the fragment size excised by this restriction enzyme are indicated. (B) Schematic representation of the targeted disruption of the PbTRAP gene. The procedure of TRAP gene disruption is the same as in A. The probe used for Southern blot analysis in C (right) is indicated by a solid bar. The recognition sites for EcoT22i and the fragment size excised by this restriction enzyme are indicated. (C) Southern blot analysis of PbMAEBL (left) and PbTRAP gene-disrupted (right) parasites. DNAs were isolated from the intraerythrocytic stages of respective parasite populations as indicated over each lane. They were digested with the restriction enzyme that is indicated over each panel and hybridized with

the probe to the targeted gene that is indicated under each panel. By the integration of the *maebI* targeting construct, the size of the detected restricted fragments increased from 0.7 to 5.0 kbp (left). By the integration of the *trap* targeting construct, the size of the detected restricted fragments increased from 1.6 to 1.9 kbp (right). (D) Immunofluorescence microscopy of the wild-type *maebI*(+) 1 (WT, left) and the PbMAEBL gene-disrupted *maebI*(-) 1 (KO, right) sporozoite. Sporozoites were collected from mosquito midguts 18 d after an infective blood meal. They were fixed with acetone for 2 min, incubated with polyclonal antibodies against the PbMAEBL repeat region, and then conjugated with FITC with second antibodies. *maebI*(-) 1 sporozoites were not stained. The same results were obtained in all parasite populations. Arrowheads, the apical end of each sporozoite; $\times 200$. (E) Western blot analysis of PbMAEBL in *maebI*(+) 1 (WT) and *maebI*(-) 1 (KO) sporozoites. The immunoblot analysis was performed with the same antibodies as used in D. Protein bands in the wild-type parasites disappeared in the knockout parasite.

(Fig. 2, B and C) and used them in the following experiment as controls (15). *trap*(-) sporozoites are known to lose infectivity due to their loss of gliding motility (15, 19), which is characteristic of invasion by *Apicomplexan* parasites (20). Therefore, it was expected that the comparison of phenotypes between *maebI*(-) and *trap*(-) populations might provide useful information about the function of PbMAEBL. In the intraerythrocytic stage, *maebI*(-) and *maebI*(+) populations showed almost equivalent growth rates in rat blood, indicating that PbMAEBL is not required for the normal proliferation of the merozoite. This result is consistent with our observation that PbMAEBL is not produced in the merozoite stage.

To assess the role of PbMAEBL in the vector-invasive stage, anopheline mosquitoes fed on rats infected with the respective parasite populations. By immunofluorescence microscopy (Fig. 2 D), immunoelectron microscopy (unpublished data), and Western blot analysis (Fig. 2 E), PbMAEBL was not detected in the midgut sporozoites of any *maebI*(-) population. 20–24 d after an infective blood meal, the number of sporozoites in the midgut, hemocoel (body cavity), and salivary glands were separately counted (Table I). In *maebI*(-) populations, the number of midgut sporozoites per mosquito was comparable to other populations. However, only a few sporozoites, presumably contaminants from the hemolymph, were associated with the salivary glands. Conversely, the number of hemolymph sporozoites significantly increased, indicating that they were normally released from the oocysts but accumulated in the hemolymph, probably because sporozoite entry into the

salivary gland was interrupted at an early stage. When compared with PbTRAP gene-disrupted parasite populations, it is especially noteworthy that the number of salivary gland-associated sporozoites of *maebI*(-) populations was ~ 20 times smaller than that of *trap*(-) populations. Because *trap*(-) sporozoites have been reported to be associated with the gland surface by trypsin-sensitive binding (15), these results suggest that *maebI* gene disruption impairs sporozoite ability to attach to the gland surface.

MAEBL Is Not Involved in Sporozoite Gliding Motility In Vitro. Impaired motility can reduce sporozoite infectivity to the host cell. To test the possibility that MAEBL gene disruption affects sporozoite motility, the gliding motility of *maebI*(-) sporozoites was observed in vitro. As shown in Fig. 3, the hemolymph sporozoite of a *maebI*(-) population (middle) glided in a normal circle and speed, comparable to that of the *maebI*(+) wild-type populations (top), and in clear contrast with the PbTRAP gene-disrupted population that completely lost its ability to glide (C) (15). The proportion of the motile sporozoites in *maebI*(-) populations was also identical to that of the *maebI*(+) wild-type populations (Table II).

MAEBL Does Not Participate in Sporozoite Infection of the Vertebrate Host. The infectivities of *maebI*(-) sporozoites to the vertebrate host were investigated using oocyst sporozoites, which are infective to the vertebrate host with a low efficiency (21). In this experiment, infectivities were assessed by the infection rate and the prepatent days required for infected erythrocyte appearance in the rat's blood after intravenous injection. As shown in Table II,

Table I. Sporozoite Distributions in *Anopheles stephensi* Mosquitoes Infected with Wild-type, *maeb1(-)*, and *trap(-)* Populations

Parasite population	Percent of infected guts	Midgut	No. of sporozoites/mosquito	
			Hemolymph	Salivary gland
WT	ND	66,200 ± 12,300	2,867 ± 809	13,733 ± 3,130
<i>maeb1(+)</i> 1	85.7 ± 2.3	79,900 ± 8,684	5,633 ± 285	11,933 ± 1,430
<i>maeb1(+)</i> 2	85.3 ± 3.4	69,233 ± 17,120	4,000 ± 416	12,233 ± 649
<i>maeb1(-)</i> 1	ND	136,075 ± 19,900	12,575 ± 1,738	2.3 ± 0.4
<i>maeb1(-)</i> 2	ND	60,500 ± 12,040	9,667 ± 3,517	1.4 ± 0.9
<i>maeb1(-)</i> 3	96.8 ± 1.4	127,400 ± 19,400	15,267 ± 3,019	1.4 ± 0.3
<i>maeb1(-)</i> 4	97.0 ± 3.0	82,233 ± 15,070	15,333 ± 1,868	2.8 ± 0.5
<i>trap(-)</i> 1	94.7 ± 2.9	111,167 ± 10,900	9,700 ± 700	32.4 ± 4.6
<i>trap(-)</i> 2	98.0 ± 1.0	115,600 ± 3,110	7,867 ± 498	49.4 ± 13.3

Mosquitoes fed on mice infected with wild-type polyclonal populations (WT) or respective parasite populations. 20–24 d after blood feeding, sporozoites were separately collected from the salivary glands, the hemolymph, and the midgut as previously described (15). Each value is the mean with its standard error of at least three independent experiments using 30–40 mosquitoes in each experiment.

the infection rate and prepatent period of the *maeb1(-)* populations were virtually identical to those of the *maeb1(+)* populations. These results show that PbMAEBL is not required for liver cell infection, which is consistent with the observation that the sporozoite greatly reduced its micronemal content of PbMAEBL after salivary gland infection (Fig. 1 A).

Discussion

Although MAEBL has been classified as a merozoite protein, our immunological studies did not detect MAEBL in the merozoite. On the other hand, we found that the sporozoite produced this protein abundantly before the invasion of the salivary gland and greatly reduced its content after invasion. Furthermore, our MAEBL gene disruption experiments showed that MAEBL is essential for sporozoite infection of the mosquito salivary glands but not for the merozoite infection of the erythrocyte. These results lead us to conclude that MAEBL is specifically produced by the sporozoite in the oocyst and acts when it infects the mosquito salivary gland.

The immunological detection of MAEBL presence previously reported was performed with an antiserum raised against glutathione-*S*-transferase-fused MAEBL carboxyl cysteine domain. Noe et al. (18) reported that MAEBL was detected as a 120–140-kD protein in the merozoite by Western blot analysis using this antiserum. However, this size is much smaller than that estimated from the amino acid sequence (~200 kD). On the contrary, our two antibodies of distinct origins detected an ~200-kD protein in the sporozoite stage. The specificity of our antibodies was demonstrated by nonreactivity with MAEBL gene knock-out parasites (Fig. 2, D and E). The combined results strongly suggest that the antiserum to glutathione-*S*-transferase-fused carboxyl cysteine domain used by Noe et al. (18) cross reacted with a distinct protein in the merozoite, which gave the erroneous conclusion that MAEBL is produced by the merozoite.

Immunoelectron microscopic observation showed that MAEBL is localized in the microneme of the sporozoite in the oocyst. The microneme is an apical organelle involved in the invasive motility of the malarial parasite into the host cell and contains adhesive protein-like molecules that be-

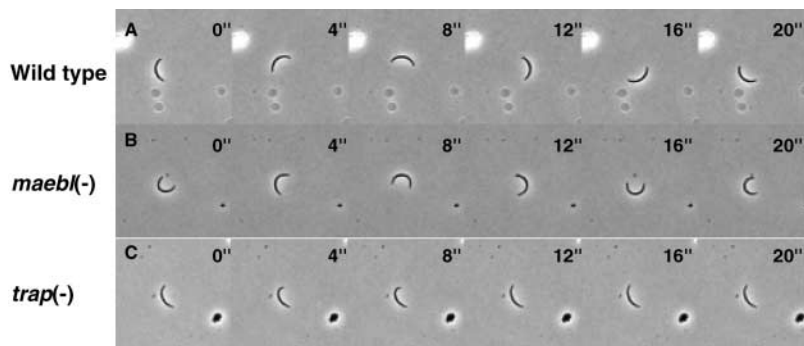


Figure 3. PbMAEBL gene-disrupted parasites, in contrast to PbTRAP-disrupted parasites, showed normal gliding motility in vitro. Sporozoites were collected from the hemolymph of mosquitoes infected with the respective parasite populations 24 d after an infective blood meal. They were kept for 2 h in 3% BSA at 4°C and then gliding on an uncoated microscope slide was observed under a phase-contrast microscope. Time lapse micrographs were recorded at 4-s intervals. ×200.

Table II. Infectivities to Rats and Proportions of Gliding Motility-positive Parasites in Wild-type, *maebl*(-), and *trap*(-) Populations

Parasite population	Percent gliding motility ^a	No. of injected midgut sporozoites	No. of infected rats ^b	Prep. period ^c	No. of injected midgut sporozoites	No. of infected rats ^b	Prep. period ^c
WT	6.1		9/15	<i>d</i> 5.2		12/15	<i>d</i> 5.2
<i>maebl</i> (+) 1	7.8	50,000	7/10	4.6	100,000	8/10	4.6
<i>maebl</i> (+) 2	7.6		5/10	5.6		9/10	4.7
Average				5.1			4.8
<i>maebl</i> (-) 1	5.9		10/10	5.1		9/10	4.7
<i>maebl</i> (-) 2	7.5	50,000	8/15	5.0	100,000	14/15	4.4
<i>maebl</i> (-) 3	7.2		4/10	5.2		10/10	4.6
<i>maebl</i> (-) 4	7.1		9/10	4.7		10/10	4.7
Average				5.0			4.6
<i>trap</i> (-) 1	0.0					0/4	—
<i>trap</i> (-) 2	0.0				500,000	0/4	—

Sporozoites were collected from mosquitoes infected with respective populations 20–24 d after an infective blood meal. For analysis of gliding motility, the hemolymph sporozoites were kept for 2 h in 3% BSA at 4°C and then gliding on an uncoated microscope was observed under a phase-contrast microscope. For analysis of infectivity, midgut sporozoites were injected into 3-wk-old Wistar rats.

^aOnly sporozoites displaying typical circular gliding were counted.

^bNumber of infected rats/number of rats injected with midgut sporozoite suspension.

^cNumber of days between sporozoite injection and detection of at least one erythrocytic stage upon a 10-min examination of a Giemsa-stained blood smear. The value is the mean value of successful infection.

long to protein families conserved among different malarial species or stages, such as the TRAP and DBL families. MAEBL is structurally related to the DBL family, which is a family of merozoite microneme proteins that play a critical role in merozoite commitment to the erythrocyte by forming a tight junction between the merozoite apical surface and the erythrocyte receptors (11, 22). The structural relation to these proteins and its essential role in salivary gland infection suggest that MAEBL may mediate sporozoite migration into the salivary gland by binding to salivary gland receptors.

Both MAEBL and TRAP are microneme proteins of the sporozoite in the oocyst and are involved in its infection of the salivary gland. However, our gene disruption experiment revealed clear phenotypic differences between MAEBL gene knockout sporozoites and TRAP gene knockout sporozoites. TRAP gene knockout sporozoites lost infectivity to both the mosquito salivary gland and the liver cell of the vertebrate host, whereas MAEBL gene knockout sporozoites lost infectivity only to the salivary gland. Furthermore, TRAP gene knockout sporozoites became nonmotile, whereas MAEBL gene knock out sporozoites showed normal gliding motility *in vitro*. These differences indicate that these molecules play distinct roles in

the host cell invasion machinery of the sporozoite. It has been suggested that TRAP is necessary for sporozoite invasion into the host cell, most likely by transmitting motility powered by an actomyosin motor across the parasite plasma membrane (19). On the other hand, normal motility on a glass slide displayed by the MAEBL gene knockout sporozoite suggests that MAEBL is necessary to change the motility observed *in vitro* into the gliding movement *in vivo* through direct interactions with the salivary gland. This possibility would be supported by its structural relation to the merozoite adhesive proteins, which interact directly with erythrocyte surface receptors and determine the host specificity of the merozoite.

Another important phenotypic difference observed was the number of sporozoites collected from the salivary glands of infected mosquitoes. As previously reported, some sporozoites are collected from the salivary glands of mosquitoes infected with TRAP gene knockout parasites. In our experiments, ~30–50 sporozoites per mosquito were obtained on average. Sultan et al. (15) reported that such sporozoites were not in the gland lumen but were associated with the gland surface by trypsin-sensitive binding, indicating that TRAP gene knockout sporozoites irreversibly committed to salivary gland invasion by forming a

junction with the salivary gland, but their migration was arrested in the following step due to loss of motility. In contrast, the number of sporozoites collected from the salivary glands almost decreased to zero in the MAEBL gene knockout parasite. This suggests that MAEBL gene-disrupted sporozoites become unable to form a junction with the salivary gland and therefore commit to salivary gland invasion. It has been reported that the first step of sporozoite invasion into the salivary gland is migration into the space between the basal lamina of the gland and the basal plasma membrane of the salivary gland cells (3). According to our recent electron micrographic study, sporozoites form a clear, tight junction when they glide into this space (unpublished data). Thus, MAEBL might participate in sporozoite commitment to the salivary gland in this invasion step. Additional investigations including the identification and localization of MAEBL ligands are required to confirm this hypothesis.

In conclusion, we have demonstrated that MAEBL is a microneme protein that is produced by sporozoites in the oocyst and is essential for the sporozoite infection of mosquito salivary glands. We suggest that MAEBL is an attachment protein involved in sporozoite entrance into the salivary gland by binding to specific salivary gland surface receptors and provide the molecular basis to understanding the specific interactions between the malarial parasite and the mosquito salivary gland.

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