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 into the mosquito and released into the body cavity of the mosquito by oocyst rupture. They then selectively invade the salivary gland. There, they become infectious 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 lectin that recognize the salivary gland surface carbohydrates inhibited 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 the recently proposed expanded merozoite adhesive protein family, named the ebl family, which includes DBL family proteins and putative erythrocyte binding proteins encoded by

*Abbreviations used in this paper: DBL, Duffy binding-like; Pb, Plasmodium berghei; TRAP, thrombospondin-related adhesive protein.
After the removal of tissue fragments by centrifugation at 18, tissues were gently ground in the medium to release sporozoites. Trifugation at 5,000 for 3 min, sporozoites were collected from the supernatant by centrifugation. 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 CO2, 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% O2, and 5% CO2. Mature schizonts were purified from the cultured blood by density gradient using Nycroprep 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. 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.

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**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 μg/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 μg/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 (Hamatsu Photonic System) and Adobe Photoshop.

**Immunoelectron 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 μg/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 the same buffer, and then rinsed an additional five times in PBS. For nuclear staining, DAPI (0.02 μg/ml final concentration) was added to the secondary antibody solution. Immunofluorescence micrographs were obtained with a HITACHI H-800 transmission electron microscope 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'-GCCGAGCTCTTTATTTGCTTCTGATT3' and 5'-GGCCGATCTCCTTATCCAAAGTTCATCAGAAAAGT3'; and 5'-GGCTCGAGGATGAATATAATGAAGATTTCTTA3' and 5'-GGCGGATTCCAGATGAACTTGTTTCTTATT3'. These fragments were subcloned into either side of the selectable marker gene in pHbusescript 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'-GGCGAGCTCTGTA-
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 P. berghei 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. 1A, left). In contrast, staining was greatly reduced in sporozoites from the salivary gland (Fig. 1A, middle). Merozoites were not stained by either antibody (Fig. 1A, right). In immunoelectron microscopy, PbMAEBL was detected in the micronemes of mature sporozoites in the oocyst (Fig. 1B, left). On the other hand, neither antibody showed any specific affinity against the apical organelles of merozoites, including rhotories where MAEBL has been reported to be localized (Fig. 1B, right) (18). By Western blot analysis, PbMAEBL was detected as an ∼200-kD protein in oocyst sporozoite preparations but not in the merozoite stage (Fig. 1C). 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.

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. 2A 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.
Essential Function of MAEBL for Malaria Transmission

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 sliding motility of maebl(−) sporozoites was observed in vitro. As shown in Fig. 3, the hemolymph sporozoite of a maebl(−) population (middle) glided in a normal circle and speed, comparable to that of the maebl(+) wild-type populations (top), and in contrast to the PbTRAP gene-disrupted population that completely lost its ability to glide (C) (15). The proportion of the motile sporozoites in maebl(−) populations was also identical to that of the maebl(+) wild-type populations (Table II).

MAEBL Does Not Participate in Sporozoite Infection of the Vertebrate Host. The infectivities of maebl(−) 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,
the infection rate and prepatent period of the \textit{maebl} (−) populations were virtually identical to those of the \textit{maebl} (+) 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 knockout 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-

<table>
<thead>
<tr>
<th>Parasite population</th>
<th>Percent of infected guts</th>
<th>Midgut</th>
<th>No. of sporozoites/mosquito</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hemolymph</td>
</tr>
<tr>
<td>WT</td>
<td>ND</td>
<td>66,200 ± 12,300</td>
<td>2,867 ± 809</td>
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<td>85.7 ± 2.3</td>
<td>79,900 ± 8,684</td>
<td>5,633 ± 285</td>
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<td>\textit{maebl} (+) 2</td>
<td>85.3 ± 3.4</td>
<td>69,233 ± 17,120</td>
<td>4,000 ± 416</td>
</tr>
<tr>
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<td>ND</td>
<td>136,075 ± 19,900</td>
<td>12,575 ± 1,738</td>
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<tr>
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<td>60,500 ± 12,040</td>
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<td>127,400 ± 19,400</td>
<td>15,267 ± 3,019</td>
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<td>94.7 ± 2.9</td>
<td>111,167 ± 10,900</td>
<td>9,700 ± 700</td>
</tr>
<tr>
<td>\textit{trap} (−) 2</td>
<td>98.0 ± 1.0</td>
<td>115,600 ± 3,110</td>
<td>7,867 ± 498</td>
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</table>

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.

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**Table 1. Sporozoite Distributions in Anopheles stephensi Mosquitoes Infected with Wild-type, maebl(−), and trap(−) Populations**

![Image](image-url)

**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.
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 knockout 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

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

<table>
<thead>
<tr>
<th>Parasite population</th>
<th>Percent gliding motility</th>
<th>No. of injected midgut sporozoites</th>
<th>No. of infected rats</th>
<th>Prep. period</th>
<th>No. of injected midgut sporozoites</th>
<th>No. of infected rats</th>
<th>Prep. period</th>
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<tr>
<td>WT</td>
<td>6.1</td>
<td>9/15</td>
<td>5.2</td>
<td>d</td>
<td>12/15</td>
<td>5.2</td>
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<td>7.8</td>
<td>50,000</td>
<td>4.6</td>
<td>100,000</td>
<td>8/10</td>
<td>4.6</td>
<td>4.7</td>
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<td>7.6</td>
<td>5/10</td>
<td>5.6</td>
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</tr>
<tr>
<td>Average</td>
<td></td>
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<td>5.1</td>
<td></td>
<td></td>
<td>4.8</td>
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</tr>
<tr>
<td>maebl(−) 1</td>
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<td>10/10</td>
<td>5.1</td>
<td>9/10</td>
<td>4.7</td>
<td>4.7</td>
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<tr>
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<td>8/15</td>
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<td>14/15</td>
<td>4.4</td>
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<td>7.2</td>
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<td>100,000</td>
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<td>maebl(−) 4</td>
<td>7.1</td>
<td>9/10</td>
<td>4.7</td>
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<tr>
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<td>4.6</td>
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<tr>
<td>trap(−) 1</td>
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<td>0/4</td>
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<td>trap(−) 2</td>
<td>0.0</td>
<td></td>
<td>500,000</td>
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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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