Cell-Passage Activity Is Required for the Malarial Parasite to Cross the Liver Sinusoidal Cell Layer

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Liver infection is an obligatory step in malarial transmission, but it remains unclear how the sporozoites gain access to the hepatocytes, which are separated from the circulatory system by the liver sinusoidal cell layer. We found that a novel microneme protein, named sporozoite microneme protein essential for cell traversal (SPECT), is produced by the liver-infective sporozoite of the rodent malaria parasite, Plasmodium berghei. Targeted disruption of the spect gene greatly reduced sporozoite infectivity to the liver. In vitro cell invasion assays revealed that these disruptants can infect hepatocytes normally but completely lack their cell passage ability. Their apparent liver infectivity was, however, restored by depletion of Kupffer cells, hepatic macrophages included in the sinusoidal cell layer. These results show that malarial sporozoites access hepatocytes through the liver sinusoidal cell layer by cell traversal motility mediated by SPECT and strongly suggest that Kupffer cells are main routes for this passage. Our findings may open the way for novel malaria transmission-blocking strategies that target molecules involved in sporozoite migration to the hepatocyte.

Introduction

Malaria is one of the most devastating infectious diseases in the world, killing more than 1 million people per year. Malaria is transmitted by bites of infected mosquitoes that inject sporozoites under the skin. The first obligatory step for these parasites to establish infection in humans is migration to hepatocytes, where they proliferate and develop into the erythrocyte-invasive form (Sinnis 1996). This liver-invasive stage has been demonstrated as a promising target for antimalarial strategies that aim to establish sterile immunity against the malarial parasite (Nussenzweig et al. 1967; Hoffman et al. 1996). However, the mechanisms underlying the parasite’s liver infection are largely unknown. In particular, it has been controversial how sporozoites reach the hepatocytes that are separated from blood circulation by the liver sinusoidal layer. The routes the sporozoites use to cross this layer, the modes of motility on which their migration is based, and the molecules of the parasite involved in this process are poorly understood.

Malarial parasites develop into sporozoites in the mosquito midgut and then invade the salivary gland, where they wait to be transferred to the mammalian host (Menard 2000). Once injected by mosquito bites under the skin, sporozoites enter the blood circulation and are carried to the liver by the bloodstream (Sinnis and Nussenzweig 1996; Menard 2000; Mota and Rodriguez 2002). In the liver, they are thought to be arrested on the inner surface of the liver sinusoidal vein and then leave the vein and infect the hepatocytes by crossing the sinusoidal wall (Sinnis and Nussenzweig 1996). This wall is a single-cell layer mainly composed of sinusoidal endothelial cells and Kupffer cells, which are hepatic macrophages. Several models have been proposed to explain how sporozoites cross this layer. Some authors proposed that sporozoites infect hepatocytes after crossing the liver endothelial cell through fenestrations in this cell (Vanderberg and Stewart 1990), but these openings are too small for sporozoites to freely pass through (Mota and Rodriguez 2002). Other authors have suggested that Kupffer cells are gates for sporozoites to access hepatocytes, based on the ultrastructural observation that sporozoites were found inside Kupffer cells shortly after intravenous inoculation (Mota and Rodriguez 2002). This Kupffer cell hypothesis, however, has not been convincingly demonstrated, because other tools for probing into this event were lacking. Furthermore, the observation that the sporozoites in Kupffer cells sometimes have a vacuole around them makes the conclusion uncertain. Some authors have proposed that sporozoites are passively engulfed by Kupffer cells and then carried to the hepatocyte (Meis et al. 1985), and some have proposed that this migration is based on active motility accompanied by vacuole formation (Pradel and Frevert 2001).

The malarial parasite has no locomotory organelles such as flagella or cilia. Motility of the host-invasive stages of the malarial parasite, including the sporozoite, is dependent on secretion of micronemes that are organelles occupying the cytoplasm of the parasite (Sultan 1999; Menard 2001). Micronemal components, which may include several attach-
ment proteins, are secreted from the apical pore during parasite movement and are translocated backwards along the parasite cell surface by actomyosin motors of the parasite. This surface movement is believed to generate traction for parasite-invasive motility.

Salivary gland sporozoites display three modes of motility in vitro dependent on secretion of micronemes (Mota and Rodrigues 2002). One mode is gliding motility on a solid surface, which can be observed as circular movement on a glass slide, probably representing gliding motility on the cell surface. The other two are cell-invasive motilities: cell-infection and cell-traversal motility (Mota et al. 2001; Kappe et al. 2003). Cell-infection motility is accompanied by vacuole formation and is followed by parasite development into exoerythrocytic forms (EEFs). Cell-traversal motility, on the other hand, involves plasma-membrane disruption and is followed by migration through the cytoplasm and eventual escape from the cell. Recently, Mota et al. (2002) revealed that this type of cell-invasion motility can be identified by conventional cell-wound assay. According to the observation that passage through some hepatocytes by this motility precedes hepatocyte infection, they proposed the hypothesis that this motility is necessary for sporozoites to be activated for hepatocyte infection (Mota et al. 2002). However, the role of this motility in liver infection remains unclear.

Aiming at identification of molecules involved in sporozoite infection, we screened an expressed sequence tag (EST) database of the salivary gland sporozoite of a rodent malarial parasite, Plasmodium berghei. In this paper, we report a novel microneme protein, named SPECT (sporozoite microneme protein essential for cell traversal), which is specifically produced by the liver-infective sporozoite and is essential for the sporozoite’s cell-passage ability. By using spect-disrupted parasites, we show that cell-passage ability of the sporozoite plays a critical role in malarial transmission to the vertebrate host and is required for sporozoites to access hepatocytes by traversal of the liver sinusoidal cell layer. In addition, we provide a model of sporozoite liver infection, which suggests an answer to the question of how sporozoites reach the hepatocytes.

Results
Identification of cDNA Encoding SPECT from P. berghei Salivary Gland Sporozoite EST Database

Sporozoites acquire the ability to infect the mammalian liver after infection of the mosquito salivary glands (Sultan et al. 1997), indicating that novel protein synthesis for liver infection begins in this stage (Matuschewski et al. 2002). To search for malarial genes involved in liver infection, we screened an EST database of P. berghei salivary gland sporozoites. We assembled 3,825 ESTs, obtained 502 contigs, and screened them for genes encoding secretory proteins or membrane-associated proteins, which may participate in host-parasite interactions. This screening was started from the contigs containing a high number of ESTs, since the number of ESTs may correlate with the expression levels of the respective genes. In this process, we identified a contig composed of ten ESTs, encoding a putative secretory protein of 241 amino acids (Figure 1A). The expected molecular mass for the N-terminal signal sequence-processed form of this protein was 25 kDa. We named this protein SPECT (sporozoite microneme protein essential for cell traversal), since it is essential for sporozoite passage through a host cell, as described later.

Southern blot analysis showed that the spect gene is a single-copy gene (data not shown). Sequence analysis of the spect gene identified four introns (data not shown). A computer search of Plasmodium genome databases (Carlton et al. 2002; Gardner et al. 2002) revealed that this gene is conserved through several Plasmodium species. The orthologous protein in P. falciparum, the clinically most important human malaria parasite, shared 45.6% sequence identity with P. berghei SPECT (Figure 1B).

SPECT Is Produced Specifically by Salivary Gland Sporozoites and Localized in Micronemes

The expression profile of this gene in the malarial life cycle was investigated. Immunofluorescent analysis in all host-invasive stages showed that SPECT production was restricted to sporozoites in the salivary gland (Figure 2A). It is noteworthy that SPECT is not detected in sporozoites in the midgut, because this expression profile strongly suggests...
that SPECT is specifically involved in liver infection. Western blot analysis revealed SPECT as a 22 kDa protein in salivary gland sporozoites, but not in midgut sporozoites (Figure 2B), confirming that SPECT is produced after invasion into the salivary gland. Immunoelectron microscopy showed that SPECT is localized in the sporozoite to micronemes that are secretory organelles occupying the cytoplasm (Figure 2C). Micronemes are common to motile stages of *Plasmodium* parasites and play a central role in host-invasive motility (Sultan 1999; Menard 2001). Taken together, these results indicate that SPECT plays a role in the liver-invasive motility of the sporozoite.

**SPECT Plays an Important Role in Sporozoite Infection of the Host Liver**

To investigate the function of SPECT protein, we generated *spect*-disrupted parasites by homologous recombination (Figure 3A). The *spect* disruptants were selected by the antimalarial drug pyrimethamine and were separated from wild-type parasites by limiting dilution. Disruption of the *spect* locus was confirmed by Southern blot analysis (Figure 3B). To exclude the possibility that the *spect*-disrupted populations obtained were derived from a single clone, two independently obtained *spect*-disrupted populations (*spect*1 and *spect*2) were used in the following experiments.

In the intra-erythrocytic stage, SPECT gene disruption did not affect parasite proliferation, as the growth rates in rat blood were almost the same in the *spect*-disrupted and wild-type populations (data not shown). Furthermore, disruption of the gene did not affect parasite development in the mosquito vector, as numbers of sporozoites residing in the midgut and in the salivary glands were similar in the *spect*-disrupted and wild-type populations (Table 1).
of infected erythrocytes, was measured in the exponential growth period (from 3.5 d to 5 d after inoculation). It is thought that the parasitemias reflect the liver infectivity of the respective parasite populations, since the growth rates of their intraerythrocytic stages are similar (shown by the parallel slopes of the increase in parasitemia in Figure 4). Based on the average parasitemia at 3.5 d after inoculation of 30,000 sporozoites, the liver infectivities of the two disruptant strains were estimated to be 15- and 28-fold lower, respectively, than that of the wild-type. These results are consistent with the observation that the parasitemias after injection of 30,000 disruptant sporozoites were lower than that from 3,000 wild-type sporozoites.

The liver infectivity was also evaluated by the number of early EEFs. Frozen sections of the rat liver were prepared 24 h after sporozoite injection and EEFs were counted by immunofluorescence microscopy. As shown in Figure 4B, EEFs were approximately 30-fold decreased by spect gene disruption. This reduction rate agrees well with that estimated by parasitemia. These results indicate that SPECT plays a role in the process of sporozoite invasion into the liver.

SPECT Is Essential for Sporozoite Cell-Passage Ability

Localization of SPECT in micronemes indicates its involvement in the invasive motility of the sporozoite. The motility of spect-disrupted sporozoites was investigated by three in vitro assays corresponding to three modes of motility of the sporozoite. First, we checked gliding motility on a solid surface, which is essential for sporozoite infectivity. Most disruptants displayed a typical circular movement, and the proportion of motile sporozoites was almost identical in disruptant and wild-type parasites (63.6% and 67.5%, respectively), showing that their gliding motility is not affected by SPECT gene disruption. Second, we examined the ability of the sporozoites to infect hepatocytes. This was assayed by formation of EEFs in a human hepatoma cell line, HepG2 (Hollingdale et al. 1981). As shown in Figure 5A, the disruptants formed EEFs in similar numbers to the wild-type, indicating that they retain normal infectivity to the hepatocyte. Third, we examined cell-traversal ability that takes place prior to hepatocyte infection. This was estimated by the number of membrane-wounded cultured cells that were labeled by uptake of fluorescein isothiocyanate (FITC)-conjugated dextran from the medium (Mota et al. 2001). As shown in Figure 5B, the cell-wound assay using HeLa cells showed that the disruptants lost their cell-passage activity completely. The same results were obtained in HepG2 cells (data not shown). These results revealed that SPECT is specifically involved in cell-traversal ability and suggest that lack of this ability reduced liver infectivity of the disruptants.

Cell Passage Ability Is Necessary for Sporozoites to Traverse the Sinusoidal Layer Cells and to Access Hepatocytes

To access the hepatocytes, sporozoites must cross the sinusoidal layer, which separates them from the circulation. We assumed that SPECT was necessary for this process. Since Kupffer cells are major components of this layer and have been reported as the main gates for sporozoite access to the hepatocyte, we prepared Kupffer cell-depleted rats by intravenous injection of liposom-encapsulated dichloromethylene diphosphonate (Cl2MDP) (Vreden et al. 1993; van Rooijen and Sanders 1994) and tested them for infection by disruptant and wild-type sporozoites. As shown in Figure 6A, infectivities of spect-disruptants assessed by parasitemia were increased by 22- and 53-fold by Kupffer cell depletion and, as a result, became equal to that of the wild-type. The numbers of early EEFs detected in the liver sections were also almost identical in wild-type and spect-disrupted parasites (Figure 6B). These results show that the disruptants’ loss of infectivity is localized at the sinusoidal cell layer and that the cell-passage ability of the sporozoite is necessary to cross this layer and, specifically, the Kupffer cells.

Table 1. SPECT Disrupted Parasites Develop Normally into Sporozoites and Invade the Salivary Gland in the Mosquito Vector

| Parasites Population | Number of Sporozoites per Mosquito | Number of Sporozoites per Mosquito |
|----------------------|-----------------------------------|-----------------------------------|
| spect(−)1            | 141,367 ± 36,205                  | 14,136 ± 1,778                    |
| spect(−)2            | 69,800 ± 9,825                    | 16,233 ± 4,138                    |
| Wild-type            | 86,674 ± 8,454                    | 19,396 ± 3,33                     |

Mosquitoes were fed on mice infected with spect(−) parasite populations or wild-type polyclonal populations. Sporozoites were collected separately from the midgut and the salivary glands of mosquitoes 24–28 d after feeding and then counted. Each value is the mean of the number with its standard error from three independent experiments.

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Figure 4. Targeted Disruption of spect Results in Reduction of Sporozoite Infectivity to the Liver

(A) The salivary gland sporozoites of each parasite population were injected intravenously into five rats. The parasitemia of each rat was checked by a Giemsa-stained blood smear after inoculation on the days indicated. The average parasitemia after inoculation of 30,000 sporozoites was significantly low in disruptant populations, whereas their growth rates in the blood were essentially the same as the wild-type. The numbers of parasites inoculated were as follows: 30,000 spect(−)1(open circles), 30,000 spect(−)2(open triangles), 30,000 wild-type (filled circles), and 3,000 wild-type (filled squares). Values shown represent the mean parasitemia (± SEM) of at least three rats.

(B) The salivary gland sporozoites (500,000) of wild-type or spect-disrupted parasites were inoculated intravenously into 3-wk-old rats. After 24 h, the livers were fixed with paraformaldehyde and frozen. The number of EEFs on each cryostat sections was estimated by indirect immunofluorescence analysis using anti-CS antiserum. Values shown represent the mean number of EEFs per square millimeter (± SEM) of at least three rats.

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Discussion

It has been reported that the *Plasmodium* sporozoite has the ability to traverse cultured cells rapidly (Mota et al. 2001), but the role of this process in liver infection has remained unclear. On the other hand, it is poorly understood how the sporozoite migrates from the circulatory system to the hepatocyte. In this paper, we address these issues using a gene-targeting technique. We have shown that the cell-traversal activity of the sporozoite is necessary for it to leave the circulatory system by crossing the liver sinusoidal cell layer. These results are the first to reveal the role of cell-traversal activity in malarial transmission.

In vitro cell invasion assays showed that *spect*-disrupted sporozoites completely lose cell passage activity, but preserve normal infectivity to the hepatocyte (see Figure 5). These results clearly demonstrated that these two cell-invasion activities are independent of each other. This conclusion contradicts the hypothesis proposed by Mota et al. (2002) that cell passage activates the sporozoite for hepatocyte infection. They assumed that sporozoites traverse some hepatocytes before infecting a hepatocyte and that this passage alters their mode of cell invasion from passage to infection (Mota et al. 2002). Our results, however, demonstrated that lack of previous cell passage has no influence on the infectivity to hepatocytes. This independence was confirmed in vivo by the result that disruptants and wild-type showed the same liver infectivities in Kupffer cell-depleted rats (see Figure 6). Therefore, sporozoites may change their mode of invasive motility according to other factors, which remain to be elucidated. We suppose that secretion of the micronemal contents during gliding on the cell surface might be one such factor, since this motility may precede hepatocyte infection as discussed below.

Our results indicate that the liver sinusoidal barrier is not perfect, since a small proportion of the *spect*-disrupted sporozoites can infect the liver (see Figure 4). It is supposed that this layer may have a few openings and the disruptants can migrate through them by gliding along the epithelial cell surface. In Kupffer cell-depleted rats, on the other hand, both disruptants and wild-type may migrate through the numerous gaps created among the endothelial cells, resulting in elimination of the phenotypic difference. Since Kupffer cells constitute approximately 30% of the sinusoidal cells (Bouwens et al. 1986), their depletion from this layer may leave
Sporozoites migrate to the space of Disse through the Kupffer cells. [1] The sporozoite (Sp) in the circulatory system is sequestered to the sinusoidal endothelial cell (EC) by specific recognition of the cell surface or glycosaminoglycans extending from the hepatocytes (He) through fenestration. [2] The sporozoite begins to glide on the epithelial cell surface. [3] Encountering a Kupffer cell (KC), the sporozoite ruptures the plasma membrane, passes through the cell, and enters into the space of Disse. Thus, the sporozoite gains access to hepatocytes. This step requires SPECT. [4] The sporozoite infects a hepatocyte with formation of a vacuole and develops into EEF in the hepatocyte.

(Right) An alternative route to the hepatocyte. A small number of sporozoites, which find gaps in the sinusoidal layer while gliding, migrate to hepatocytes directly through the openings without need for cell passage and infect the hepatocytes. Likewise, in Kupffer cell-depleted rats, both wild-type and spect-(-) sporozoites can enter hepatocytes through numerous gaps present between the sinusoidal endothelial cells.

Materials and Methods

Parasite preparations. Female 6–10-wk-old BALB/c mice (Japan SLIC, Inc., Hamamatsu, Japan) infected with the P. berghei ANKA strain were prepared by peritoneal injection of infected blood that was stored at -70°C. For the purification of sporozoites, infected mosquitoes were dissected 24–28 d after the infective blood meal. The salivary glands and midgut were separately collected in medium 199 on ice and then gently ground to release the sporozoites. Ookinetes and erythrocytic-stage parasites were prepared as described previously (Yuda et al. 1999; Kariu et al. 2002).

Genomic Southern blot hybridization. Genomic DNA of P. berghei parasites (2 μg) was digested with CiaI, EcoRI, EcoT22I, HindIII, or XbaI, separated on 1.2% agarose gel and then transferred to a nylon membrane. DNA fragments were amplified by PCR using genomic DNA as template with the following primers: 5'-TGGGCAATTTTG-3' and 5'-TTTCATTGTGTTAAACGATAAGTG-3'. They were labeled with [32P]dCTP and used as probes.

Antibody preparation and Western blot analysis. Recombinant SPECT without signal sequence was expressed as a glutathione S-transferase (GST)-fusion protein using the pGEX 6p-1 system (Amersham Bioscience, Uppsala, Sweden). The recombinant protein was purified with a GST column and used for immunization of rabbits. Specific antibodies were affinity purified using a N-hydroxy succinimidyl-activated column (Amersham Bioscience).
coupled with recombinant SPECT protein. For CS antigen production, the peptide DPPPPNPADPNPN, corresponding to the repeat region, was conjugated to keyhole limpet hemocyanin as a carrier and used for the immunization of rabbits. Western blot analysis was performed as described previously (Karui et al. 2002).

**Immunofluorescence microscopy and immunoelectron microscopy.** Immunofluorescence was performed with 3% paraformaldehyde and 1% glutaraldehyde for 15 min on ice. After embedding in LR gold resin (London Resin Company Ltd., London, United Kingdom), ultrathin sections were incubated with anti-SPECT antibodies and then with secondary antibody conjugated to gold particles (15 nm diameter) (AuroProbe, Amersham Pharmacia Biotech, Uppsala, Sweden). The samples were examined with a Hitachi H-800 transmission electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV.

**Targeted disruption of the spect gene.** For construction of the targeting vector, two fragments of the spect gene were amplified by PCR using mouse spect cDNA as templates with primer pairs 5'-CGGGGATCCCGTCAATATGTTAATTGGGCTAGCCA-3' and 5'-GGCGGATCCCGTGAATTCACATTTCAATGTTAATTGGTTCCTATTTATCATTTTAAAATGTGTTT-3' and 5'-CGGGCGGATCCCGTCAATATGTTAATTGGGCTAGCCA-3'. These fragments were cloned into either side of the selectable marker gene in pBluescript (Strategene, La Jolla, California, United States) (Mota et al. 2001). Before each inoculation, sporozoites were checked for their ability to glide in vitro to confirm that they contained over 60% motile sporozoites. Parasitemia was calculated.

**Evaluation of sporozoite infectivity to rats.** Sporozoites collected from mosquito salivary glands were suspended in medium 199 and from mosquito salivary glands were suspended in medium 199 and injected intravenously into 3-wk-old female Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) (n = 5). Before each inoculation, sporozoites were checked for their ability to glide in vitro to confirm that they contained over 60% motile sporozoites. Parasitemia was calculated.

**Measurement of the number of EEFs in the infected liver.** Sporozoites (5.0 × 105) were intravenously inoculated into a 3-wk-old female Wistar rat. After 24 h, the liver was perfused with PBS followed by 4% paraformaldehyde. The liver was further fixed in 4% paraformaldehyde for 6 h and frozen in liquid nitrogen. Cryostat sections (20 µm) were prepared from the left lobe and fixed in acetone for 2 min on a glass slide. The EEFs were detected by immunofluorescence staining using rabbit anti-CS antisera and FITC-conjugated secondary antibody. At least 12 sections were examined under an Olympus (Tokyo, Japan) BX60 fluorescence microscope (200× magnification) and the number of EEFs per square millimeter was calculated.

**EEF development assay in vitro.** The EEF formation assay was performed as described previously (Hollingdale et al. 1981) with minor modifications. HepG2 cells (5.0 × 105) were plated in 8-well chamber slides. Sporozoites (3.0 × 105 or 5.0 × 105) were suspended in 100 µl of complete medium and added to this culture. After 2 h, the media were replaced with 400 µl of fresh complete medium supplemented with 4% paraformaldehyde for 24 h with medium changed twice a day and were fixed in acetone for 2 min. The EEFs were detected by immunofluorescence staining as described above. The number of EEFs in one-fifth of the area of each well was counted under an Olympus BX60 fluorescence microscope (200×).

**Cell-traversing activity assay.** The traversing activity of the sporozoite was examined using a standard cell-wounding and membrane repair assay (Mota et al. 2001). HepG2 cells (2.5 × 105) or HeLa cells (5.0 × 105) were inoculated into 8-well chamber slides (Nunc Inc., Naperville, Illinois, United States). Sporozoites were added 2 d later to cells for 1 h in the presence of 1 mg/ml FITC-labeled dextran (10,000,000, lysine-fixable; Molecular Probes, Inc., Eugene, Oregon, United States). The cells were incubated for an additional 3 h in complete culture medium and fixed with 4% paraformaldehyde in PBS. The number of FITC-positive cells was counted under a fluorescence microscope.

**Depletion of rat Kupffer cells.** For depletion of Kupffer cells, 3-wk-old female Wistar rats were injected intravenously with 120 µl of LPS (Sigma Chemical Co., St. Louis, Missouri, United States). The control treatment was 0.9% NaCl. After 48 h, sporozoites were inoculated into a tail vein and the parasitemia was checked by Giemsa-stained blood smears. CLMDP liposomes were prepared as described elsewhere (van Rooijen and Sanders 1994). Elimination of Kupffer cells was confirmed by immunoperoxidase staining after liver perfusion with PBS followed by fixation with 4% paraformaldehyde in PBS. The number of FITC-positive cells was counted under a fluorescence microscope.

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**Conflicts of interest.** The authors have declared that no conflicts of interest exist.

**Author contributions.** TI, YC, and MY conceived and designed the experiments; TI, KY, and MY performed the experiments; TI and MY analyzed the data. TI and MY wrote the paper.

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