Transformation of Sporozoites into Early Exoerythrocytic Malaria Parasites Does Not Require Host Cells

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Abstract

Malaria parasite species that infect mammals, including humans, must first take up residence in hepatic host cells as exoerythrocytic forms (EEF) before initiating infection of red blood cells that leads to malaria disease. Despite the importance of hepatic stages for immunity against malaria, little is known about their biology and antigenic composition. Here, we show that sporozoites, the parasites’ transmission stage that resides in the mosquito vector salivary glands, can transform into early EEF without intracellular residence in host hepatocytes. The morphological sequence of transformation and the expression of proteins in the EEF appear indistinguishable from parasites that develop within host cells. Transformation depends on temperature elevation to 37°C and serum. Our findings demonstrate that residence in a host hepatocyte or specific host cell–derived factors are not necessary to bring about the profound morphological and biochemical changes of the parasite that occur after its transmission from vector to mammalian host.

Key words: Plasmodium • liver stage • hepatocyte • temperature • axenic culture

Introduction

Malaria is caused by obligate intracellular protozoan parasites of the genus Plasmodium. Plasmodia have a complex life cycle that alternates between mosquito vector and vertebrate host. Transmission occurs when infected mosquitoes bloodfeed and inoculate sporozoites. After entering the bloodstream, sporozoites are quickly transported to the liver where they extravasate and subsequently invade hepatocytes. Within host hepatocytes, the elongate sporozoites transform into spherical hepatic stages (exoerythrocytic forms [EEF]). EEF are the obligatory intermediary tissue stage between the parasites’ residence in the mosquito vector and the initiation of red blood cell infection by merozoites in the mammalian host (1). Transformation is a radical cellular remodeling process (2). It becomes first discernible by a bulbous expansion of the sporozoite that contains the single nucleus. This bulb progressively enlarges until the banana-shaped sporozoite is completely absorbed. The spherical EEF grow, undergo multiple rounds of nuclear division (3), and finally differentiate into thousands of first generation merozoites. It is not known what signals trigger transformation into hepatic stages and what factors are essential to support it.

Plasmodium hepatic stages are exceedingly difficult to study because they are rare and preparations are always contaminated with hepatocyte material. Therefore, little is known about their antigenic repertoire with only a few EEF-expressed proteins identified (4). However, defining antigens expressed in early liver stages is an important goal for preerythrocytic vaccine development (5). Complete development of hepatic stages of Plasmodium berghei, a model Plasmodium species that infects rodents, has been achieved in vitro using the hepatoma cell line HepG2 as a host cell (6). This in vitro system, together with a line of P. berghei that expresses green fluorescent protein (GFP; reference 7), facilitates microscopic monitoring of invasion and subsequent EEF development. In HepG2 culture infections, we frequently observed extracellular sporozoites that transformed into EEF and were still detectable after 24 h in culture. Thus we investigated the potential of sporozoites to transform into early hepatic stages in the absence of host hepatocytes.

Materials and Methods

Parasites. 4–5-d-old female Anopheles stephensi mosquitoes were blood-fed on anesthetized Swiss CD-1/ICR mice infected with wild-type P. berghei strain NK65 or a GFP-expressing P. berghei NK65 line. Rodents were assayed for high levels of parasitemia and the abundance of gametocyte-stage parasites capable
of exflagellation. Afterwards, the infective bloodmeal mosquitoes were maintained at 21°C, 80% humidity. On day 10 after feeding, the mosquitoes were dissected in RPMI 1640, and the isolated midguts were examined for the infection rate. Only mosquito cages having at least 70% of infected mosquitoes were kept for further analysis. Mosquitoes were rinsed in 70% ethanol for 5 min and washed twice in sterile medium to reduce contamination. Salivary glands were dissected in sterile DMEM (BioWhittaker) containing 500 U/ml penicillin–streptomycin and 1.25 µl/ml fungizone (Sigma-Aldrich). The glands were disrupted, and the sporozoites were isolated and counted in a hemocytometer.

Transformation Medium and Quantification of Transformation. Sporozoites were suspended in DMEM containing 2 mM l-glutamine and 4.5 g/liter glucose, and supplemented with 10% FBS (HyClone Laboratories), 500 U/ml penicillin–streptomycin, and 1.25 µl/ml fungizone. 5 × 10⁴ sporozoites were inoculated per well of 8-well chamber slides (Labtek) and maintained at 37°C in 5% CO₂. Initially, the slides were coated with Matrigel (Becton Dickinson) at 100 µg/cm² density to improve the development. Matrigel improved the attachment of the parasite to the slide, but it was not essential for transformation. To determine transformation rates for various conditions, slides were examined by fluorescence microscopy at 40X using P. berghei–expressing GFP. EEF were scored as completely transformed only when they appeared completely spherical with no visible sporozoite remnants. Each count was performed in triplicate on three independent experiments done with sporozoites isolated from different batches of infected mosquitoes. As a control, P. berghei was cultivated in HepG2-A16 cells (HB 8065; American Type Culture Collection).

Immunofluorescence Assays. Transformation was confirmed by immunofluorescence assay using monoclonal antibodies against heat shock protein 70 (HSP70), circumsporozoite protein (CSP), thrombospodin-related anonymous protein (TRAP), and a rabbit polyclonal antibody against myosin A tail domain interacting protein (MTIP), an inner membrane complex–associated protein. Parasites were removed from culture chambers, fixed in 2% paraformaldehyde, washed in PBS, concentrated by centrifugation, suspended in 1% FBS/PBS-diluted primary antibody, and incubated at 37°C for 1 h. After three washes, the samples were incubated in 40 µg/ml 6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. After three washes in 1% FBS/PBS, the parasites were transferred onto 12-well slides and dried at room temperature. Slides were incubated with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes) and diluted 1:250 in 1% FBS/PBS for 1 h at 37°C.

Immunoblot. 8 × 10⁵ salivary gland sporozoites and the content of chambers inoculated with the same number of sporozoites and maintained for 24 h at 37°C were collected. The transformation rate for this batch was 13%. Samples were suspended in 10 µl of SDS loading buffer and incubated for 5 min at 70°C. Antigen extracts were subjected to 7% SDS-PAGE and transferred onto a polyvinylidifluoride membrane. The membrane was blocked overnight at 4°C in PBS containing 5% dry milk, and incubated for 1 h with anti-HSP70 antibody. The membrane was washed in PBS/0.1% Tween 20 and incubated with 1 h with 1:5,000 diluted horseradish peroxidase–labeled anti–mouse IgG (Amersham Biosciences) and developed with enhanced chemiluminescence (ECL; Amersham Biosciences).

Reverse Transcripase (RT)–PCR. 5 × 10⁵ P. berghei sporozoites were inoculated on 12-well plates and placed at 37°C or 22°C. 24 h after inoculation, the content of each well was collected, and total RNA extraction was performed using the RNasy Mini kit (Qiagen). RNA was treated with DNase I (Invitrogen) to remove contaminating genomic DNA. First strand cDNA synthesis was done using an RT-PCR kit (PE Applied Biosystems) with random hexamers. PCR amplification was done using oligonucleotide primers specific for the P. berghei A type rRNA (sense, 5'-CGAGAATCTGGCTCCTGGCTC-3'; antisense 5'-CTAGCTCCTGTGACTGCTTTATC-3'). This amplified a specific 460-bp fragment. P. berghei S type rRNA was amplified using oligonucleotide primers (sense, 5'-CAGCTCTCTGCTCCTGTC-3'; antisense 5'-CTACCTGCTTAAAGAGATAGTT-3'). This amplified a specific 457-bp fragment. PCR was done for 30 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 3 min.

PCR amplification for merozoite surface protein-1 (MSP-1) was done using oligonucleotide primers (sense, 5'-GGAGAAATGCAGTGGCTCAGGGA-3'; antisense 5'-TTTAAATGCCTCAAGAATATTATTTTCT-3'). This amplified a specific 120-bp fragment. PCR amplification for HEP17 was done using oligonucleotide primers (sense, 5'-AAAAAGGAAGACATC-CATTCC-3'; antisense 5'-TGTATCTCCTCCAGTGAAGAA-3'). This amplified a specific 120-bp fragment. PCR was done for 30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 3 min.

Results and Discussion

To test if sporozoites can transform in the absence of host cells, they were cultured without HepG2 cells in microchamber slides coated with basement membrane extract (Matrigel) and DMEM containing 10% FBS for 4, 10, and 24 h at 37°C. Microscopic examination showed that after 4 h, sporozoites frequently developed the transformation bulb (Fig. 1 A) that was also typically observed during early intracellular transformation (2). At 10 h, the extracellular transformation had proceeded further to intermediate EE-like forms displaying a more extended bulb and further retraction of the armlike remnants of the sporozoite cell body (Fig. 1 B), closely resembling the progression of transformation observed during intracellular transformation (3, 8).

At 24 h, ~13% of sporozoites had transformed into completely spherical EE-like forms (Fig. 1, C–E, and Table I). This rate of transformation exceeds the published rates of transformation achieved in HepG2 cells, which range from 3 to 8% (6, 9) and the rates of transformation in HepG2 cells observed by us (~5%, unpublished data). EE-like forms that were cultured without HepG2 cells were similar in size (6–10 µm) and morphology when compared with EEF that grew within HepG2 cells (Fig. 1, F–H). No sporozoites were detectable in 24-host cell–free cultures, indicating that untransformed sporozoites did not survive.

We tested if expression of EEF proteins is comparable between EEF that undergo intracellular development and EE-like forms that develop without host cells, using antibodies against HSP70, CSP, TRAP, and MTIP. Although HSP70 of Plasmodium is highly expressed in EEF and erythrocytic stages of the parasite, it is barely detectable in sporozoites (10–12). Therefore, HSP70 expression can be used to follow the transformation of sporozoites into hepatic stages. After 6 h, early extracellular EE-like forms.
identified by the transformation bulb showed increasing HSP70 expression (Fig. 2 A). After 18–24 h, completely spherical EE-like forms had developed that showing intense HSP70 staining (Fig. 2 B). When cultures were incubated for 48 h, most of these early EE-like forms seemed not to develop further, but a few EE-like forms showed significant increase in size and compartmentalization of HSP70 staining (Fig. 2 C). This might correspond to the cytoplasmic compartmentalization observed in intracellular EEF at this time point (13). HSP70 staining patterns closely resembled the staining patterns of intracellular EEF developing inside HepG2 cells. Up-regulation of HSP70 expression was confirmed by immunoblot analysis (Fig. 2 D). HSP70 expression was barely detectable in protein extracts of 800,000 salivary gland sporozoites. However, expression increased dramatically when the same number of parasites where cultured in the host cell–free system for 24 h (13% transformation rate). HSP70 expression might be induced by temperature elevation, the transformation

Table I. Host Cell–free Transformation into Hepatic Stages

| Conditions | Spherical trophozoites | Transformation |
|------------|------------------------|---------------|
| 37°C       | 6,725.34               | 13.43         |
| 22         | 34.67                  | 0.07          |
| 22 → 37°C  | 9,656                  | 19.32         |
| 37 w/o FBS | 346.67                 | 0.69          |

124-h cultures, except 22 → 37°C, shift is 48 h; (Materials and Methods for culture conditions).

2Average number from three independent experiments. Parasites were scored as completely transformed when they appeared spherical with no sporozoite remnants visible. (Fig. 1 D).

3Percentage of transformation was calculated based on the 5 × 10⁴ sporozoites used for culture.



Figure 1. Transformation of Plasmodium sporozoites into EEF does not require host cells. P. berghei sporozoites and EEF express green fluorescent protein enabling the visualization of live parasites. (A–E) Sporozoites cultured at 37°C without HepG2 cells transform through the classic sequence of morphological changes into EE trophozoites. (A) After 4 h of culture, many parasites exhibit the transformation bulb (arrows), which is the first morphological indication of transformation. (B) After 10 h of culture, parasites have progressed further in transformation, as indicated by enlargement of the transformation bulb and concurrent retraction of the amnionlike sporozoite remnants (arrows). The insets show transforming parasites at higher magnification. After 24 h, (C–E) spherical trophozoites have developed that are indistinguishable from (F–H) trophozoites that developed within host cells. Micrographs A–D, F, and G are GFP fluorescence images; E and H are differential interference contrast images (DIC) showing the same trophozoites as in D and G, respectively. Bars, 10 µm.

Figure 2. EEF cultured without host cells express key antigens. (A–C) Different stages of EEF development show increased expression of HSP70. (A) Early 6-h EEF showing the typical transformation bulb. HSP70 expression is mostly localized to the bulb. (B) Spherical 18-h EEF trophozoite showing intense HSP70 expression. (C) Large 48-h EEF showing compartmentalization of HSP70 staining. Micrographs on the right show the corresponding DIC images. (D) Western blot analysis of HSP70 expression. Sporozoites (Spz) express HSP70 at barely detectable levels. Host cell-free EEF, cultured for 24 h, show a dramatic increase in HSP70 expression. (E) Spherical 24-h EEF trophozoite showing surface expression of circumsporozoite (CS) protein. (F) Spherical 24-h EE trophozoites do not express TRAP. Nuclei in E and F are visualized with DAPI. (G) EEF grown for 24 h without host cells express transcripts encoding MSP-1 (left) and HEP17 (right). RT-PCR with gene-specific oligonucleotide primers amplified specific fragments of 120 base pairs (bp). Controls are PCR reactions without prior RT. Bars, 5 µm.
event itself, or both. However, because parasites kept at 37°C for 24 h either transformed or died, it was not possible to investigate this further.

CSP, the major sporozoite surface protein (14), is involved in sporozoite host cell recognition. It is also expressed on the plasma membrane of early EEF (15, 16) and might fulfill additional functions during hepatic stage development. Robust CSP surface expression was detected on EE-like forms cultured in the absence of host cells for 24 h using a monoclonal antibody specific for CSP (Fig. 2 E; reference 17). In contrast, TRAP, a sporozoite micronemal protein involved in host cell invasion (18), was not detectable on spherical EE-like forms at this time (Fig. 2 F). The loss of TRAP expression coincides with the disappearance of micronemes after sporozoite transformation in vivo (3). We also determined that P. berghei EE-like forms cultured in the absence of host cells for 24 h expressed transcripts (Fig. 2 G) encoding MSP-1 (19), and the hepatic and erythrocytic stage protein 17 (HEP17; reference 20).

Apicomplexan zoites, including the Plasmodium sporozoite, are delimited by a tri-laminar pellicle consisting of a plasma membrane and two closely aligned inner membranes that form the inner membrane complex (IMC). After hepatocyte invasion, the sporozoite plasma membrane becomes the EEF plasma membrane; however, the IMC is disassembled and is not detectable at ~30 h after invasion (3). Dismantling the IMC might be essential for entry of the parasite into the trophic phase because its rigidity could interfere with growth. MTIP localizes to the IMC of sporozoites (21) and can serve as a marker to follow the loss of the IMC during hepatic stage development. Dual fluorescence assays with antibodies against CSP and MTIP showed that the IMC was lost in EE-like forms grown without host cells (Fig. 3, A–C). IMC loss was sometimes simultaneous with a first round of nuclear division but these events did not occur in a synchronized fashion (Fig. 3, B and C). The IMC loss in host cell–free EEF closely resembled its loss in intracellular EEF (Fig. 3 D). Lactacystin (10 µM), a highly specific inhibitor of proteasome activity,
inhibited host cell–free transformation by ∼70% (unpublished data), indicating that EE-like transformation was a proteasome-dependent process. This was consistent with its previously observed inhibitory effect on sporozoite transformation into intracellular EEF (22). Together, the data show that, in the absence of intact host cells or host cell–derived components, sporozoites transform into EEF that undergo nuclear division and express proteins similar to early EEF that develop within host cells. The host cell–free culture system provides a unique opportunity to study what environmental factors directly govern transformation. The stimulus for transformation might be a shift in temperature experienced by the parasite during transmission. To test this, we incubated sporozoites at either 22°C (temperature of mosquito vector) or 37°C (temperature of mammalian host) for 24 h. In contrast to sporozoites cultured at 37°C, sporozoites cultured at 22°C initiated transformation (revealed by the occurrence of transformation bulbs), but rarely developed into completely spherical EEF (Fig. 3 E and Table I). Therefore, a shift from low to high temperature is not necessary for initiation of the transformation process but high temperature is required for complete transformation of sporozoites into EEF. Interestingly, parasites kept at 22°C for 24 h could still develop into spherical EEF when the temperature was subsequently shifted to 37°C for 24 h (Table I), and this occurred with higher efficiency of transformation (∼19%) than in cultures directly incubated at 37°C. Thus, parasites kept at low temperature remained viable, experiencing a reversible arrest of transformation. In addition, we determined that the presence of serum was also critical for transformation (Table I). Without serum, few parasites transformed into spherical EEF and most parasites were not detectable in 37°C cultures after 24 h.

Switching occurs between the expression of different types of ribosomal RNA (rRNA) at transition points in the Plasmodium life cycle (23). One such transition is the transformation of sporozoites into EEF when rRNA expression switches from S-type in sporozoites (S for sporozoite) to A-type in EEF and ensuing blood stages (A for Asexual; reference 24). We determined rRNA expression in the host cell–free transformation system at 22°C and 37°C after 24 h of culture by reverse transcriptase-PCR using oligonucleotide primers specific for either A-type or S-type. Expression of A-type rRNA was detected in host cell–free EEF cultures at both temperatures (Fig. 3 F). Thus, expression of A-type rRNA was not dependent on temperature elevation, however, it seemed to increase with the elevated temperature. S-type rRNA was detectable at 22°C but was not detectable at 37°C, confirming the rRNA switch (Fig. 3 F). This indicated that either the elevated temperature repressed expression or that the S-type is under control of a cold-stimulated promoter. Thermoregulation of rRNA gene expression was described recently to also occur in the parasites’ blood stages (25).

A central tenet of Plasmodium transmission is that the invasive sporozoite must penetrate a host hepatocyte and take up intracellular residence to initiate development into EEF. However, we have shown that in principle, sporozoites do not require an intact host cell or any specific host cell–derived factors to transform into EEF. Our results suggest that in vivo, sporozoites might initiate the transformation program before invasion as soon as they enter the mammalian bloodstream. After sporozoites enter the bloodstream, they rapidly sequester in the liver and invade hepatocytes within minutes (26). This rapid homing ensures that transformation occurs only after invasion of a suitable host cell. Although sporozoites can transform into EEF without host cells, intracellular residence is likely to be essential for the parasites’ trophic phase when host cell–derived factors are needed for further growth. Plasmodium merozoites can transform into trophozoites and develop outside a red blood cell (27) only in the presence of red cell extract, indicating that merozoite transformation and subsequent parasite development was dependent on some yet undefined host cell factors.

More than 50 yr after their discovery (1), the biology and antigenic repertoire of EEF remains largely unstudied, mainly due to the small number that can be obtained in vivo and in vitro and the technical challenge of separating the intracellular EEF from uninfected hepatocytes and surrounding host hepatocytes. Sterile, protective immunity against malaria in humans and animal models has only been achieved by immunization with radiation-attenuated sporozoites (5, 28, 29). Irradiated sporozoites invade hepatocytes and transform into early EEF, but remain arrested at this stage (30, 31). Thus, it is the antigens expressed in early EEF that confer the sterile, protective immunity (32). Therefore, identification of these antigens must be regarded as the most important goal toward development of a preerythrocytic malaria vaccine (5, 33, 34), and this might be facilitated by a host cell–free transformation system.

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