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(Citation)
Molecular and Biochemical Parasitology,236:111261-111261

(Issue Date)
2020-03

(Resource Type)
journal article

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/90007288

Molecular & Biochemical Parasitology 236 (2020) 111261

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

journal homepage: www.elsevier.com/locate/molbiopara

Observation of morphological changes of female osmiophilic bodies prior to *Plasmodium* gametocyte egress from erythrocytes

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ARTICLE INFO

Keywords:
Plasmodium
Female osmiophilic body
Activated gametocyte
Egress
G377

ABSTRACT

Plasmodium parasites cause malaria in mammalian hosts and are transmitted by *Anopheles* mosquitoes. Gametocytes, which differentiate from asexual-stage parasites, are activated by environmental changes when ingested into the mosquito midgut, and are rapidly released from erythrocytes prior to fertilization. Secretory proteins localized to osmiophilic bodies (OBs), organelles unique to gametocytes, have been reported to be involved in female gametocyte egress. In this study, we investigate the dynamics of OBs in activated gametocytes of *Plasmodium falciparum* and *Plasmodium yoelii* using the female OB-specific marker protein, G377. After activation, female gametocyte OBs migrate to the parasite surface and fuse to form large vesicles beneath the parasite plasma membrane. At the marginal region of female gametocytes, fused vesicles secrete contents by exocytosis into the parasitophorous vacuole space, prior to parasite egress *via* the break-down of the erythrocyte membrane. This is the first detailed description of how proteins are transported through osmiophilic bodies.

Malaria parasite transmission from an infected mammalian host to the mosquito vector requires the uptake of gametocytes, intraerythrocytic sexual precursor cells, into the mosquito midgut. Mature male and female gametocytes are activated in the mosquito midgut by environmental changes such as temperature drop, increased pH, and the presence of mosquito xanthurenic acid. Activated gametocytes egress from erythrocytes and participate in fertilization and zygote formation. Numerous proteins are involved in erythrocyte egress in *Plasmodium berghei* and *Plasmodium falciparum*, including male development-1/ protein of early gametocyte 3 (MDV1/PEG3), gamete egress and sporozoite traversal (GEST), and plasmodial perforin-like protein 2 (PPLP2) [1–4]. Among them, MDV1/PEG3 and GEST are localized to osmiophilic bodies (OBs), specialized secretory vesicles that are present only in gametocytes and play important roles in gametocyte egress from the erythrocyte [1,2]. While OBs are scattered in the cytoplasm of gametocytes, it is reported that rupture and immediate vesiculation of the parasitophorous vacuolar membrane (PVM) occurs, and then a limited area of the erythrocyte surface membrane opens when activated gametocytes are released from the erythrocyte [5]. G377, which is specifically expressed in female gametocyte OBs, is demonstrated to be important for the formation of OBs, although it is not essential for parasite egress from erythrocytes [6–13]. G377 tagged with a fluorescent protein has been shown to lead to redistribution of the protein from a dispersed to a membrane associated pattern in female gametocytes [9,14,15]. However, there is no detailed report on changes in the distribution and morphology of OBs that occur following gametocyte activation and up to their release from erythrocytes. Here, we investigated the dynamics of OBs in activated female gametocytes by immunoelectron microscopy (IEM) using as a marker the protein G377, which is specifically localized in the OBs of female gametocytes.

Recombinant proteins were synthesized to generate antibodies against G377 of *P. falciparum* (PF3D7_1250100) and *P. yoelii* (PY17X_1465600). A fragment encoding PfG377 (amino acid positions [aa] 666–1146) [6] was amplified from PfNF54 genomic DNA by PCR, using primer pairs PfG377-flagB-XhoI-F (5′-gagactcgagCCTGAACCATG GCCTCTTGATG-3′) and PfG377-flagB-BamHI-R (5′- gagaggatcctcaTCA ATTTGTTTTTGTTTGGCTAGTCAAATC-3′). A fragment encoding PyG377 (amino acid positions [aa] 2004–2204) [12] was amplified

Abbreviations: GST, gluthathione S-transferase; IEM, immunoelectron microscopy; OB, osmiophilic body; PVM, parasitophorous vacuole membrane; PPM, parasite plasma membrane

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https://doi.org/10.1016/j.molbiopara.2020.111261
Received 14 November 2019; Received in revised form 21 January 2020; Accepted 22 January 2020
Available online 22 January 2020

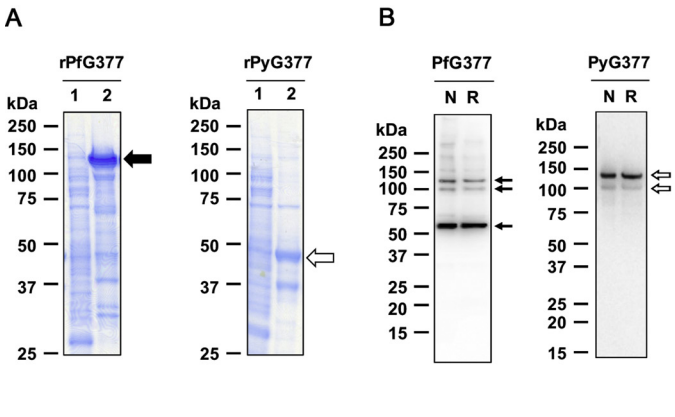

Fig. 1. (A) SDS-PAGE analysis of G377 proteins expressed by the wheat germ cell-free system. Protein mixtures were separated by SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue. The total reaction mixture from the wheat germ cell-free system is shown in lane 1, and affinity-purified proteins in lane 2. Closed arrow, PfG377-GST; open arrow, PyG377-GST. (B) Western blot analysis using antibodies against G377 of *P. falciparum* and *P. yoelii*. Proteins from approximately 1×10^5 gametocytes per lane were separated by SDS-PAGE under non-reducing (lane N) and reducing (lane R) conditions. Closed arrow, PfG377; open arrow, PyG377. (C) Subcellular localization of female osmiophilic bodies indicated by G377 in *P. falciparum* and *P. yoelii* gametocyte-infected erythrocytes. Acetone-fixed non-activated and activated gametocytes were incubated with rabbit anti-PfG377 antibodies or mouse anti-PyG377 antibodies (shown in green). DIC, differential interference contrast microscopy image. Bar, 5 μm.

from Py17X genomic DNA by PCR, using primer pairs PY17X_1465600-XhoI-F1 (5′-gagactcgagAAATTAATAGAACCATTTGTA CCTATTAG-3′) and PY17X_1465600-BamHI-R1 (5′-gagaggatcctcaTTC TTTATTTCCTGTTTTATTTAATTTAT-3′). Amplified DNA fragments of pfg377 or pyg377 were independently inserted between the XhoI and BamHI sites of plasmid pEU-E01-GST-TEV-MCS-N2 (CellFree Sciences, Matsuyama Japan). The recombinant proteins were expressed, as fusion proteins having a gluthathione S-transferase (GST) tag at their N-terminus, using a wheat germ cell-free system (CellFree Sciences). After synthesis, recombinant PfG377 and PyG377 fragments were affinity purified by passage through a glutathione-Sepharose 4B column (GE Healthcare, Camarillo, CA, USA) and eluted with elution buffer (40 mM reduced glutathione, 50 mM Tris-HCl, 300 mM NaCl, 200 mM imidazole, 2 % glycerol, and pH 8.0) (Fig. 1A). To generate antiserum against the PfG377 fragment, a Japanese white rabbit was immunized subcutaneously with 250 μg of purified recombinant protein with Freund's complete adjuvant, followed by two immunizations using 250 μg of purified recombinant protein with Freund's incomplete adjuvant. All immunizations were done at 3-week intervals and antiserum was collected 14 days after the last immunization (Kitayama labes, Ina, Japan). Mouse antiserum against PyG377 was produced by immunizing a BALB/c mouse with 20 μg of recombinant protein per immunization according to the above protocol. The proteins of gametocyte-rich samples from PfNF54 or Py17X were resuspended in reducing or non-reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and then boiled at 97 °C for 5 min, followed by separation by electrophoresis on a 12.5 % polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were transferred to 0.2 μm polyvinylidene fluoride membranes (ATTO). Membranes were incubated with Blocking One (nacalai tesque, Kyoto, Japan) followed by immunostaining with rabbit antiserum against PfG377 (1:2000 dilution) or mouse antiserum against PyG377 (1:100 dilution) as the primary antibody. The membranes were then probed using HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (GE Healthcare) and visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA) on a LAS 4000 luminescent image analyzer (GE Healthcare). The relative molecular masses of the proteins were estimated by reference to Precision Plus Protein Standards (Bio-Rad, Hercules, CA, USA). PfG377 and PyG377 were detected as triple or double bands, respectively, under reducing and non-reducing conditions as reported (Fig. 1B) [8,12]. The expression pattern of G377 in gametocytes was confirmed by IFA. Infected blood smears containing gametocytes were fixed on glass slides with ice-cold acetone for 5 min and blocked with PBS containing 5 % non-fat milk (PBS milk) at 37 °C for 1 h. They were then incubated with rabbit anti-PfG377 antiserum 1:5000 dilution or mouse anti-PyG377 antiserum 1:100 dilution at 37 °C for 1 h, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG antibodies (Invitrogen) as a secondary antibody (1:500 dilution) at 37 °C for 30 min. The IFA staining pattern of antibodies against PfG377 or PyG377 on non-activated gametocytes shows a granular appearance characteristic of the osmiophilic bodies [7]. In activated gametocytes, the fluorescent dots were relocated to be loosely associated to the parasite membrane as reported [9] (Fig. 1C). From these findings, it was confirmed that antisera prepared against PfG377 or PyG377 specifically recognize the cognate proteins in parasites.

Morphological changes of OBs following activation of female gametocytes were examined by IEM. To activate gametocytes, infected erythrocytes were mixed with ookinete culture medium (RPMI1640 containing 25 mM HEPES, 24 mM sodium bicarbonate, 100 μM xanthurenic acid, 50 mg/L hypoxanthine, and 10 % human serum for *P. falciparum* or 20 % fetal bovine serum for *P. yoelii*, pH 7.6) and incubated at 24 °C for 30 or 10 min. For IEM analysis, gametocyte-infected erythrocytes before or after activation were fixed for 30 min on ice in a mixture of 1 % paraformaldehyde and 0.2 % glutaraldehyde in 1 × HEPES buffer (pH 7.05). Fixed specimens were dehydrated and embedded in LR-White resin (Polysciences Inc, Warrington, PA, USA). Ultrathin sections on a grid were blocked in PBS containing 5 % non-fat milk and 0.01 % Tween 20 (PBS-MT) then incubated at 4 °C overnight with rabbit anti-PfG377 antiserum 1:1000 dilution or mouse anti-PyG377 antiserum 1:100 dilution in PBS-MT. After washing with PBS containing 0.4 % Block Ace Powder (DS Phama Biomedical, Tokyo, Japan) and 0.01 % Tween 20 (PBS-BT), the grids were incubated at 37 °C for 1 h with goat anti-rabbit IgG or goat anti-mouse IgG antibodies conjugated to 15 nm gold particles (BBI International, Minneapolis, MN, USA) diluted 1:20 in PBS-MT as described [10]. Samples were examined with a transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan). In IEM observation, OBs with gold particles demonstrating the localization of G377 were observed scattered in the cytoplasm of both *P. falciparum* and *P. yoelii* female gametocytes (Fig. 2A, B, G, H). These OBs in female gametocytes showed a characteristic oval-shape as reported [12]. In activated *P. falciparum* female gametocytes, relatively large vesicles containing gold particles appear alongside female OBs (Fig. 2C, D, E). Since these large vesicles also contain G377, it is considered that they are formed by fusion of multiple OBs during female gametocyte activation. In addition, an image was observed in which a part of a large vesicle containing PfG377 was fused with the parasite plasma membrane (PPM) of an activated female gametocyte and the contents being released outside the parasite, possibly into the PV space (Fig. 2F). In the activated female gametocytes of *P. yoelii*, large vesicles containing PyG377, which appear to be fused OBs, were observed in close contact with PPM (Fig. 2I, J). In the final stage of the activated female gametocyte before egress, the OB in the cytoplasm disappeared and multiple large vesicles containing PyG377 were

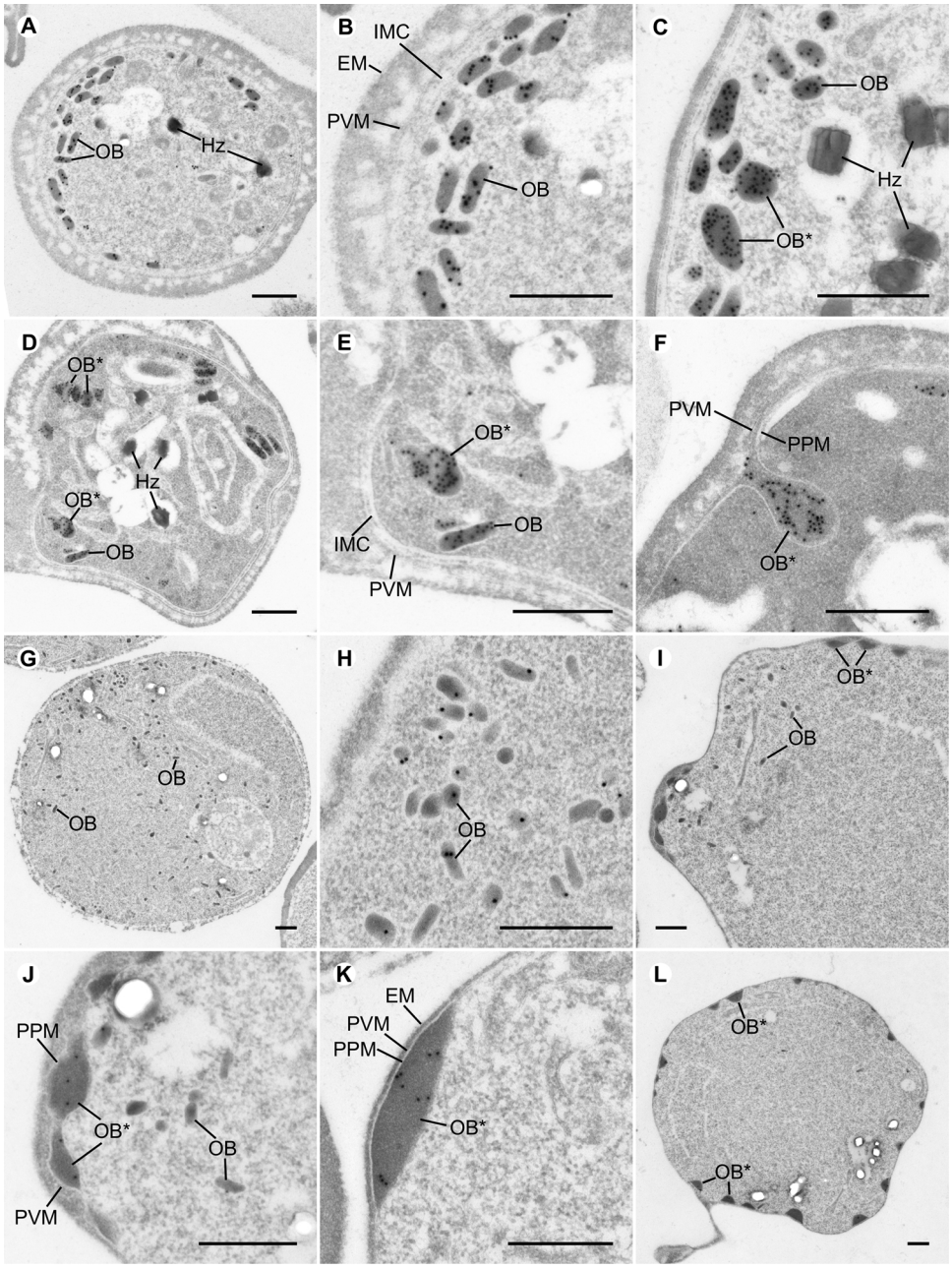

Fig. 2. Morphological changes of osmiophilic bodies in activated female gametocytes of *P. falciparum* (A to F) and *P. yoelii* (G to L). (A–B) An overview image (A) and its magnified image (B) of a non-activated female gametocyte of *P. falciparum.* Gold particles indicating the localization of PfG377 are observed in osmiophilic bodies (OB) of a non-activated female gametocyte. Hz, hemozoin granule; IMC, inner membrane complex; PVM, parasitophorous vacuole membrane; EM, erythrocyte membrane. (C–F) Activated female gametocytes after 30 min incubation in activation medium. Several osmiophilic bodies fuse to form larger vesicles (OB*). An overview image of an activated female gametocyte (D) and enlargement showing the lower left part (E). Vesicles formed by fusion of osmiophilic bodies (OB*) are found near the IMC of an activated female gametocyte. (F) Membrane of a large vesicle (OB*) is fused with parasite plasma membrane (PPM), and the contents of the vesicle are ready to be secreted into the PV space or the PVM. (G, H) An overview image (G) and magnified image (H) of non-activated female gametocytes of *P. yoelii*. Female osmiophilic bodies (OB), indicated by the deposition of PyG377, are distributed in the cytoplasm of a *P. yoelii* female gametocyte before activation. (I) In activated *P. yoelii* female gametocytes, OB move to the parasite surface and fuse to form larger vesicles (OB*). (J) Enlarged picture of (I) in which female OB fuse to form OB* and localize in close contact with PPM. (K) A fused vesicle (OB*) is localized in contact with the activated female gametocyte PPM. (L) Overall picture of an activated female gametocyte showing that fused vesicles (OB*) are distributed beneath the PPM. Bars, 500 nm.

observed in contact with the PPM (Fig. 2K, L).

This study reveals that after *in vitro* activation, female OBs containing proteins required for egress migrate near the inner membrane complex and fuse (Fig. 2C, D, E). Subsequently, the contents of the fused female OBs undergo exocytosis into the PV space by membrane fusion with PPM (Fig. 2F), and are likely involved in the destruction of PVM and erythrocyte membrane essential for egress. In *P. yoelii*, the fused female OBs are in close contact with the PPM (Fig. 2J, K), whereas in *P. falciparum* the fused female OBs are located slightly away from the PPM (Fig. 2C, E). This is probably due to the presence of an inner membrane complex under the PPM of *P. falciparum* gametocytes. Previous studies have shown that several molecules localized in OB are involved in the release of activated gametocytes from erythrocytes [1,2,8]; however, there is a lack of knowledge about OB behavior from gametocyte activation to erythrocyte egress. In this study, using G377 as a female OB molecular marker, we successfully demonstrate how OBs containing egress-related proteins behave immediately following the activation of female gametocytes until just before releasing the proteins into the PV space.

CRediT authorship contribution statement

Tomoko Ishino: Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Mayumi Tachibana:** Investigation, Funding acquisition. **Minami Baba:** Investigation. **Hideyuki Iriko:** Resources. **Takafumi Tsuboi:** Writing - review & editing. **Motomi Torii:** Conceptualization, Investigation, Resources, Writing - original draft.

Acknowledgements

We thank Aki Konishi for technical support with DNA sequencing analysis and protein production and Masachika Shudo (Advanced Research Support Center, Ehime University) for technical assistance with electron microscopy. We also would like to thank Dr. Thomas Templeton for critical reading of the manuscript. This work was

supported by JSPS KAKENHI Grants JP15K08443, JP24590506, and JP25670202.

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