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PbGCβ Is Essential for *Plasmodium* Ookinete Motility to Invade Midgut Cell and for Successful Completion of Parasite Life Cycle in Mosquitoes

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When malaria parasites enter to mosquitoes, they fertilize and differentiate to zygotes and ookinetes. The motile ookinetes cross the midgut cells and arrive to the basement membranes where they differentiate into oocysts. The midgut epithelium is thus a barrier for ookinetes to complete their life cycle in the mosquitoes. The ookinetes develop gliding motility to invade midgut cells successfully, but the molecular mechanisms behind are poorly understood. Here, we identified a single molecule with guanylate cyclase domain and N-terminal P-type ATPase like domain in the rodent malaria parasite *Plasmodium berghei* and named it PbGCβ. We demonstrated that transgenic parasites in which the *PbGC*β gene was disrupted formed normal ookinetes but failed to produce oocyst. Confocal microscopic analysis showed that the disruptant ookinetes remained on the surface of the microvilli. The disruptant ookinetes showed severe defect in motility, resulting in failure of parasite invasion of the midgut epithelium. When the disruptant ookinetes were cultured *in vitro*, they transformed into oocysts and sporozoites. These results demonstrate that PbGCβ is essential for ookinete motility when passing through the midgut cells, but not for further development of the parasites.

Key words: cell motility, gene targeting, invasion, parasitology, *Plasmodium*.

Species of *Plasmodium*, the pathogenic protozoa of malaria, are transmitted by mosquitoes. Once malaria parasite-infected blood is taken into the gut lumen of mosquitoes, mature gametocytes start gametogenesis. The male gametocytes undergo rapid DNA synthesis and shed up to eight flagella in a process termed exflagellation. The gametes fertilize and transform into zygotes. The zygotes transform into motile ookinetes and traverse midgut epithelium (*1*). Before ookinetes access the midgut epithelium, they must traverse the peritrophic membrane (PM) which is secreted by midgut epithelium and is polymerized by the blood meal (*2*). It is believed that the PM surrounds the blood bolus and protects the midgut epithelium from invasive parasites and bacteria (*3*). The ookinetes secrete chitinase to degrade PM, whereby passing through PM and traversing the epithelial cell layer (*4*, *5*). After the ookinetes arrive on basal lamina, they transform into oocysts in which thousands of sporozoites are produced. The sporozoites are released into the hemocoel and invade the salivary glands where they wait for the next blood meal to take place.

Several attempts have been made to block the parasite developments in mosquitoes, and the ookinete has been considered as a target for transmission blocking of malaria parasite in mosquitoes. The two types of ookinete surface antigens, the P25/P28 family and chitinase have been suggested as transmission blocking vaccine candidates. Antibodies against the P25/P28 family and chitinase inhibit the development of oocysts when the antibodies are introduced together with infected blood into the midgut lumen (*6*). The methodology for creating transgenic mosquito is progressively developing (*7*) and is used to create antimalarial factors expressing transgenic mosquitoes (*8*). For effective intervention of malaria transmission by using transgenic mosquitoes, more knowledge of the parasite's biology is required.

Signaling mediated by cGMP regulates a variety of events such as cellular growth and contractility, sensory transduction and neurotransmission (*9*). It has been reported that cGMP plays roles in mosquito stage development of *Plasmodium* spp. cGMP, or its analogue, enhances, while guanylate cyclase inhibitor *N*-methylhydroxylamine, suppresses exflagellation of the rodent malaria parasite, *Plasmodium berghei* (*10*). It has also been reported that mature gametocytes of *P. falciparum* have membrane-associated guanylate cyclase activity which is enhanced by xanthurenic acid, known as an endogenous inducer of exflagellation (*11–13*). These data suggest that cGMP exerts crucial roles in the regulation of parasite development of *Plasmodium* at the mosquito stage.

*PfGC*α and *PfGC*β genes have been isolated and the encoded proteins have been identified as membrane-associated guanylate cyclases of *P. falciparum*. These proteins are composed of two domains: their amino-terminal regions show strong similarity with P-type ATPases, and the carboxyl-terminal regions show

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G protein-dependent adenylate cyclases, with two sets of sex transmembrane domain (C1 and C2). The C2 domain of mammalian adenylate cyclases is located in the C1 domain of the *P. falciparum* proteins and *vice versa*. Moreover, key amino acid residues in the catalytic domains show similarity to guanylate cyclases. Consistent with this, the guanylate but not adenylate cyclase activity is detected in the bacterially expressed recombinant protein corresponding to the catalytic domains of PfGCβ. Both genes are exclusively expressed in gametocyte, and PfGCβ protein is localized to the parasitophorous vacuole membrane of gametocytes (*14*). However, biological function of PfGCα and PfGCβ remain unclear. Since cGMP signaling is important for parasite development, elucidation of their biological function may shed light on the molecular mechanism of parasite development in mosquitoes.

In the present study, we searched *P. berghei* database and identified an ortholog of *PfGC*β named as *PbGC*β. To understand its role in parasite life cycle, we created transgenic *P. berghei* in which *PbGC*β gene was disrupted and performed phenotypic analysis of the mutant parasites. The PbGCβ disruptants exflagellated, fertilized and transformed into zygotes. The zygote transformed into ookinetes, as wild type parasites. However, ookinetes showed severe defect in motility and lost the ability to traverse the midgut epithelium, resulting in the complete developmental arrest. Our study revealed that PbGCβ is essential for successful completion of the life cycle in mosquitoes.

MATERIALS AND METHODS

Maintenance of Parasites and Mosquitoes—*Anopheles stephensi* (SDA 500 strain) were reared as reported previously (*15*). *P. berghei* (ANKA clone 2.34) were maintained as cryopreserved or by mechanical blood passage and regular transmission through mosquitoes. The mice infected by mosquito biting are referred to as passage 0 (P0). The infected mice from a single mechanical passage from P0 is referred as P1 and were used in the experiment.

*Identification of PbGC*β—Guanylate cyclase genes of *P. falciparum* were searched for in PlasmoDB database (http://plasmodb.org/) where the transcript is present in gametocyte stage and encoding protein sequence contains transmembrane domains. Two genes encoding guanylate cyclase matched with these criteria and one of these, MAL13P1.301 was annotated as guanylate cyclaseβ (PfGCβ). The ortholog of *P. berhgei* was identified as partial fragments in the *P. berghei* GeneDB (http://www.genedb.org/genedb/pberghei/index.jsp). The coding region of *PbGC*β cDNA was obtained by RT-PCR using total RNA from ookinete, and 5′ and 3′ cDNA ends were amplified using rapid amplification of cDNA ends (SMART^{TM}RACE, BD biosciences). Functional prediction of translated amino acid sequence was obtained by using the Pfam database (http://pfam.wustl.edu/), simple modular architecture research tool analysis (SMAT) domain search (http://bmerc-www.bu.edu), and PROSITE scan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html). Prediction of transmembrane domain was performed by using PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/), SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0. html), TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Multiple alignments of amino acid sequences were performed using the ClustalW program with the Blosum62 matrix (http://align.genome.jp/).

Targeted Disruption of PbGCβ *Gene*—5′ and 3′ regions of the *PbGC*β gene were amplified by PCR using genomic DNA as template and primer pairs GCβ–F1-*Hin*dIII (*AAGCTT*CATTTATCCAATACCCTG) with GCβ?R1-*Hin*dIII (*AAGCTT*ATTTCCAATAAGTACCC), and GCβ–F2-*Eco*RI (*GAATTC*GTGTGTTGGAGGAATTATAG) with GCβ–R2-*Bam*HI (*GGATCC*TCGTAGTTTATAATTTTGG). The PCR fragments were digested with respective restriction enzymes and ligated to pBS-DHFR (*16*) to give a targeting vector called pPbGCβ-KO. The plasmid was digested with *Cla*I and *Eco*RI to release the insert, and the DNA was introduced in the genome of *P. berghei* by electroporation. The transfection, pyrimethamine selection and dilution cloning were performed as described (*17*). The correct recombination event of the clone was confirmed by diagnostic PCR. Two primer pairs; P1-F (CAATTGGTGATGCATATGTGGC) with P1-R (ATTTATTGTTGACCTGCAGGCATGC), and P2-F (GTTGTCTCTTCAATGATTCATAAATAGTTGGACTTG) with P2-R (CACTTTTGTGAAATTGCGACTGTG) were used to verify the correct replacement of the target gene locus with the DHFR gene. A pair of primers, W-F (TAAGGAAACACCTAACG) and W-R (GGGCTGCTGTTTTACTCCA) was used to detect possible contamination of wild type (WT) or revertant parasites in a cloned parasite population. Three clones (clone A, B and C) from two independent transfection experiments were isolated and analyzed further.

Mosquito Transmission Assay—For mosquito feeding, the tail blood of WT or disruptant (KO)-infected mice was taken and the number of exflagellating male gametes was counted. Only the mice whose blood contained over 2 exflagellating centers per 10^4 RBC were used for the feeding. Female mosquitoes (4–7 days old) were fed on mice, and fully engorged mosquitoes were collected. Sixteen days after blood feeding, midguts and salivary glands were isolated. The number of oocysts in the midgut was counted and the presence of sporozoites in the salivary gland was examined.

Midgut Immunofluorescence Stainings—Female mosquitoes were fed on WT or KO parasite-infected mice, and the midguts were dissected at 21–24 h after the feeding. The midguts were immediately fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min at room temperature and permeabilized with PBT solution [0.1% (v/v) TritonX-100 and 1% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS)] for 4 h at room temperature. For detection of ookinetes, the midguts were incubated with 1:100 diluted monoclonal antibody against Pbs-21 (kindly provided by RE Sinden, Imperial College, London) overnight at 4°C and then with Alexa568-conjugated secondary antibodies (1:500 dilution in PBT, Molecular Probes) for 6 h at room temperature. The midguts were incubated with Hoechst33348 (1:1,000 dilution, Molecular Probes) and Alexa488-conjugated phalloidin (1:100 dilution, Molecular Probes) for 20 min at room temperature to visualize nuclei and actin filaments, respectively. Fluorescence images were obtained with confocal laser scanning microscope

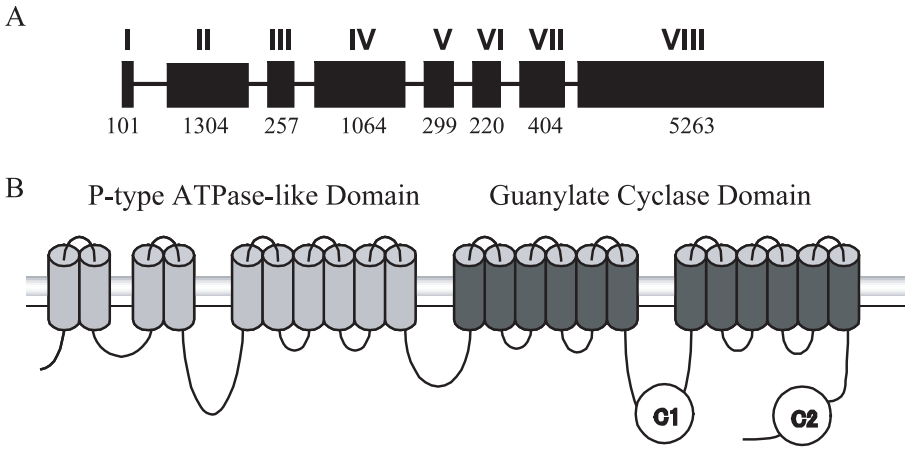

Fig. 1. **Organization of the *PbGC*β gene and the predicted topology of the PbGCβ protein.** (A) *PbGC*β gene structure. Exons are indicated as black boxes with numbers. Lower number indicates the length of each exon (bp). (B) Predicted topology of PbGCβ. The deduced amino acid sequence contains three sets of putative transmembrane (TM) regions encompassing a P-type ATPase-like domain, and two sets of 6 TM regions for the cyclase domain. C1 and C2 indicate catalytic domains of cyclase. This structural prediction is based on the model of PfGCβ proposed by Carucci *et al*. (*14*).

equipped with a standard fluorescence detector, a blue diode laser, a solid state laser, and a helium-neon (He/Ne) laser for simultaneous scanning of three fluorochromes (Digital Eclipse C1si; Nikon Co., Ltd., Tokyo, Japan). The localization of ookinetes in the midguts was examined by confocal microscopy analysis. The serial images of confocal microscopy were reconstructed using VG studio MAX for Windows version 1.1 software (Nikon visual science Co., Ltd., Tokyo, Japan) to create three-dimensional images of midgut tissues with ookinetes.

In Vitro Culture of Mosquito Stage Parasites, Measurement of Moving Velocity of the Ookinetes and Indirect Immunofluorescence Staining of Oocysts and Sporozoties—The ookinetes were obtained by *in vitro* culture of gametocytes (*18*). The ookinetes were purified by MACS magnetic column (*19*). To measure the moving velocity of ookinetes, the purified ookinetes were put on the slide glass and monitored the moving distance for 2 or 5 min by video recorder. Oocyst culture was basically carried out by the previously described method (*20*) except that S2 cells were not included in the culture. The *in vitro* generated oocysts and sporozoites were dried on slide glasses and fixed in acetone for 2 min at room temperature. The slides were blocked in 5% BSA and incubated with anti-circumsporozoite protein (CSP) antiserum (1:100) and then FITC-conjugated anti-mouse IgG antiserum (1:100). The nuclei were stained with DAPI. The CSP fused with His_6 tag at N-terminal was expressed in Sf9 cells by Bac-to-Bac Baculovirus expression system (Invitrogen). Purified recombinant protein was used for immunization of mice and the obtained antiserum was used the experiment described above.

RESULTS

Identification of PbGCβ—We searched the PlasmoDB database for guanylate cyclase genes which were expressed at the gametocyte stage and contained transmembrane domains. We found PF11_0395 and MAL13P1.301, which have been annotated as *PfGC*α and *PfGC*β, respectively (*14*). We then searched for *PbGC*β, ortholog of *PfGC*β in the *P. berghei* GeneDB and found two overlapping clones (PB000752.03.0 and PB300849.00.0). The missing initiation codon and C terminal stop codon were obtained by RACE. Sequence analysis of a series of PCR products using genomic DNA and cDNA as template revealed that the *PbGC*β gene contains 7 introns (Fig. 1A). The fully spliced transcript encodes 2,952 amino acids with a calculated molecular mass of 348.3 kDa (GenBank accession no. DQ904399). Hydrophobicity analysis indicates 22 transmembrane (TM) domains. Similarity search indicates two functional domains located in the N and C terminal regions of the PbGCβ protein; a P-type family of cation transporting ATPase and a guanylate cyclase (Fig. 1B).

The typical structure of the P-type ATPase family has two sets of two TM domains and one set of six TM domains. This character matches exactly with PbGCβ. The amino acid sequence of the P-type ATPase domain of PbGCβ shows 68% identity and 82% similarity to the corresponding domain of PfGCβ. The guanylate cyclase domain of PbGCβ contains two cassettes of 6 TM domains and catalytic domains (C1a and C2a). The first set of TM domain is followed by C1a, the second TM domain and C2a (Fig. 1B). It is known that C1a is followed by C2a in mammalian guanylate cyclases (*14*). When C1a and C2a of PbGCβ are aligned with mammalian C1a and C2a, the consensus amino acids residues of mammalian C1a are conserved in C2a of PbGCβ. Likewise, amino acid residues of mammalian C2a are conserved in C1a of PbGCβ (Fig. 2). This suggests that the position of the catalytic domains of PbGCβ is inverted as compared to mammalian cyclase. This inverted position is also observed in PfGCβ. The C1a and C2a domains of PbGCβ showed 84% and 89% identities with those of PfGCβ.

PbGCβ Gene Disruption—To understand the role of PbGCβ, we generated *PbGC*β knocked out (KO) parasites. A modified *Toxoplasma gondii* dihydrofolate reductase/thymidylate synthase (*DHFR/TS*) gene was inserted into the *PbGC*β gene by double crossover recombination, which replaces nucleotides 4,132–8,700 of the *PbGC*β gene (Fig. 3A). Diagnostic PCR confirmed correct recombination at the target gene locus (Fig. 3B). Two pairs of primers, P1-F and P1-R, and P2-F and P2-R, are KO parasite-specific. These primers gave rise to a single band only when genomic DNA of KO parasite was used, while the band was not amplified using genomic DNA of wild type parasites (WT) (Fig. 3B). Another set of primers; W-F and W-R are WT-specific and amplified a single band using genomic DNA of WT but not disruptants, indicating that the KO parasite clone was free from WT or revertants (Fig. 3B).

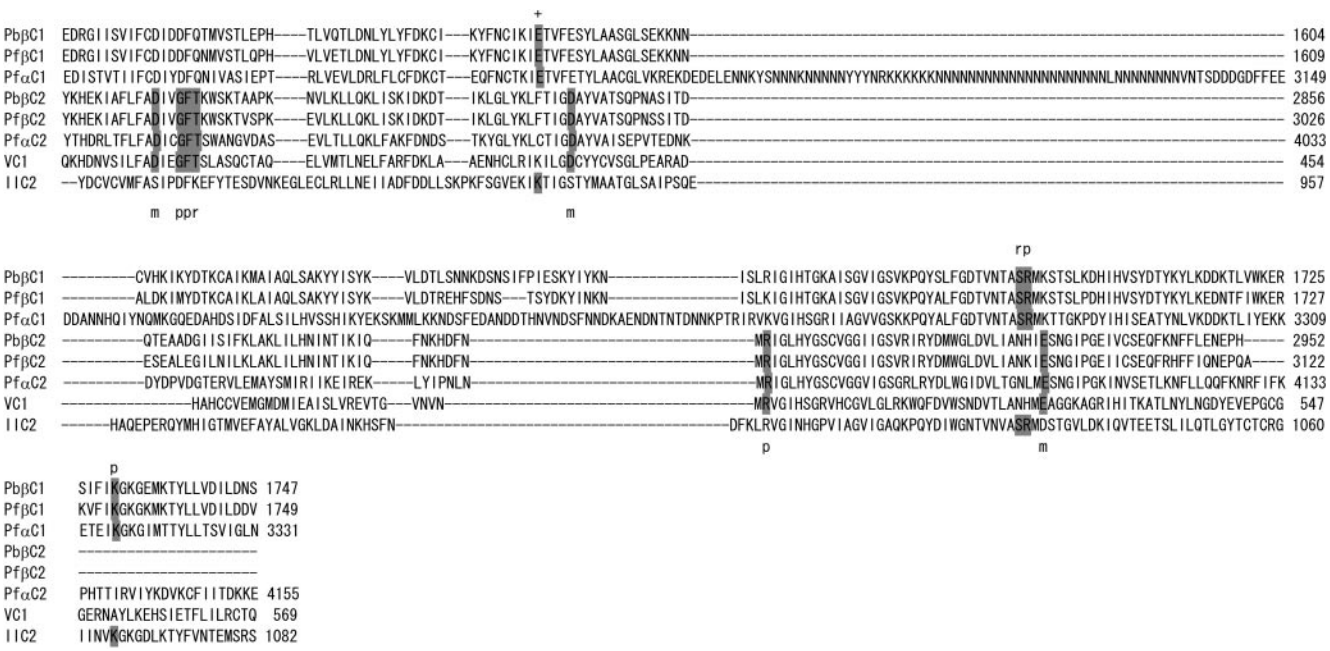

Fig. 2. **Alignment of catalytic cyclase domains (C1 and C2) of PbGCβ with corresponding domains of PfGCβ, the C1 domain of the mammalian type V adenylate cyclase (VC1) and the C2 domain of rat type II adenylate cyclase (IIC2).** The alignment reveals that the key amino acid residues conserved in the catalytic domain of C1 and C2 domain of mammalian and rat adenylate cyclase are reversely positioned in C2 and C1 of PbGCβ and PfGCβ. The information of key amino acid residues in guanylate cyclase is incorporated from Carucci *et al.* (*14*). +; purine binding, r; ribose binding, p; phosphate binding, m; divalent cation binding. The amino acid residue numbers of each sequence are given on the right.

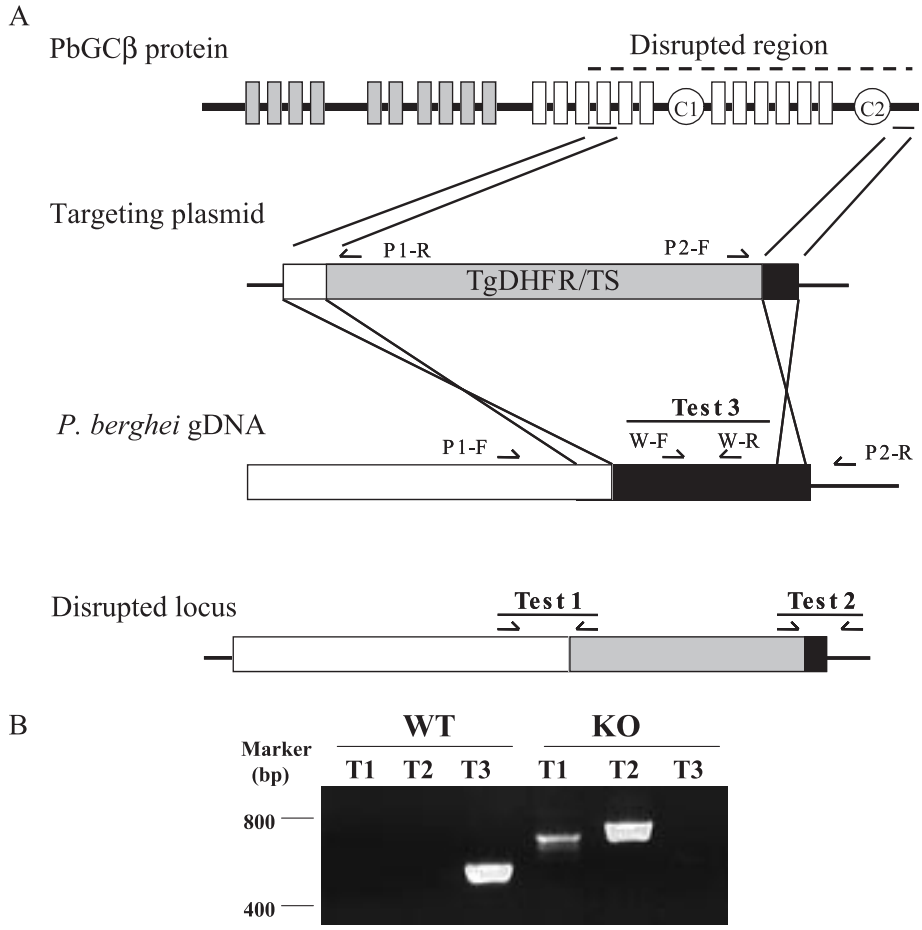

Fig. 3. **Targeted disruption of the *PbGC*β gene in *P. berghei*.** (A) Schematic representation of the PbGCβ protein and the disrupted region (top). The shaded box indicates transmembrane domain (TM) of P-type ATPase, and white box and circle are TM and catalytic sites of guanylate cyclase, respectively. The gene targeting plasmid (the second from the top) used for gene replacement by double crossover, a target gene locus of *P. berghei* (the second from the bottom) and the disrupted gene locus (bottom) are also depicted. P1-F and P1-R (Test1), and P2-F and P2-R (Test2) are replacement-specific primer pairs. W-F and W-R is a WT-specific primer pair. (B) Diagnostic PCR. Gene-targeting is confirmed by PCR using primer combinations that can only amplify a signal from the recombinant or WT locus.T1; Test 1 (P1-F and P1-R primers; recombinant specific primers), T2; Test 2 (P2-F and P2-R primers; recombinant specific primers), T3; Test 3 (W-F and W-R; WT specific primers). Test 3 confirms the absence of residual WT parasites in the recombinant parasite clone. Likewise, Test 1 and 2 give rise to a signal from the recombinant clone but not from WT.

Table 1. **Development and transmission of PbGCβ knockout parasites in *A. stephensi*.**

	Midgut		Salivary gland	Transmission
	Oocysts (+) Infected/dissected	Mean no. of oocysts per midgut (range)	Sporozoites (+) Infected/dissected	Infected (no. of mosquitoes biting)
Exp. 1				
WT	8/10	37.7 (0–108)	8/8	3/3 (8)
KO clone 1	0/35	0	0/35	0/1 (28)
Exp. 2				
WT	9/10	22.0 (0–71)	9/9	2/2 (8)
KO clone 2	0/56	0	0/56	0/1 (26)
Exp. 3				
WT	10/12	41.6 (0–154)	10/10	1/1 (8)
KO clone 3	0/60	0	0/60	0/1 (22)

Infectivity of PbGCβ Knockout Parasites—We obtained three KO parasite clones (KO1-3) from independent transfection experiments. The disruptant clones developed normally in mice and their morphological feature was indistinguishable from WT parasites. The microgametes of the KO parasite exflagellated normally, fertilized and differentiated into zygotes. The zygotes were then transformed into ookinetes *in vitro*, at an efficiency comparable to that of WT parasites (data not shown).

The mosquitoes were fed on mice infected with WT and KO parasites. The mosquitoes were dissected and the number of oocysts in the midguts was counted. Three independent experiments showed that WT parasites differentiated into oocysts and sporozoites whereas KO parasite clones (KO1-3) did not produce oocysts at all. Furthermore, no transmission from mosquitoes to mice by feeding was observed (Table 1). Thus, it appeared that PbGCβ was essential for the parasite development in mosquitoes.

Localization of Ookinetes in the Midguts—The KO parasites differentiated into ookinetes but did not produce oocysts. At least two possible explanations are conceivable for this. Either, the KO ookinetes could not penetrate the midgut epithelium and hence could not arrive to the basal lamina where WT ookinetes differentiate into oocyst. Alternatively, PbGCβ may have crucial role in oocyst formation after the KO parasites migrate to the basal lamina. To test these hypotheses, we investigated the localization of WT and KO ookinetes in the midguts. We dissected mosquitoes at 22–24 h post-feeding, fixed the midguts and removed the blood meal. After intensive washing, the ookinetes remaining in the midguts were detected by a monoclonal anti-Pbs21 antibody. Pbs21 is a surface protein expressed at high levels in *P. berghei* zygotes and ookinetes (*21*). A typical result is shown in Fig. 4A where many ookinetes were detected in the midgut fed on WT but not KO parasites (ookinetes are bright green dots in WT-1 and banana-like shape in WT-2 of Fig. 4A). Since the midguts were washed intensively before immunological detection, remaining ookinetes seems to be attaching on or invading into the midgut epithelium. The number of KO ookinetes in the midguts was less than 10% of WT ones (WT = 36.2 ± 4.0, KO = 1.2 ± 0.3. Fig. 4B), although KO parasites could differentiate into ookinetes with efficiency comparable to that of WT parasites (see Table 2). Weakly attaching ookinetes on the microvilli might be washed out during sample preparation and this could be the reason for the decrease in number of KO ookinetes in the midguts.

To further investigate the location of ookinetes in the midguts at cellular level, we scanned the midguts between microvilli (luminal side) and basement membrane (basal side) by confocal microscopy. Approximately 20 slices were taken and were merged to visualize the ookinetes with various markers at different planes of the midguts. Both WT and KO parasites could differentiate to ookinetes in the midguts (red in Fig. 5, A and B). When the midguts were analyzed from the basal side, a significant number of WT ookinetes was observed (Fig. 5C). In sharp contrast, very few ookinetes were observed at the basal side (Fig. 5D). A lateral view clearly showed that WT ookinetes invaded and resided on the basal side but KO ookinetes remained on microvilli and did not invade epithelial cells (Fig. 5, E and F). We then counted the number of ookinetes in the midguts fed on mice infected with WT and KO parasites. For this, the blood meal was not removed until sample observation. Before mounting the sample on the slide, the midguts were carefully dissected, briefly rinsed in PBS and flatted on the slide glass. Randomly selected 14 and 10 fields of each 10 midguts fed WT and KO parasites were examined for the number of ookinetes. As shown in Table 2, the number of KO ookinetes is comparable to that of WT, indicating that KO parasites fertilized and differentiated to ookinete normally in mosquitoes. Most of WT ookinetes were detected within midgut cells and basal lamina (90.6%), whereas only 4% of disruptant ookinetes were in the midgut cells, clearly showing that KO ookinetes lost the ability to invade the midgut cells.

Motility of Ookinetes—It is known that successful invasion of apicomplexan parasites requires motility which is not the classical means such as flagellum and cilia, but is called gliding motility (*22*). Since the KO parasites lost the ability to invade midgut cells, it is conceivable that this was due to lost the motility. We assessed the motility of KO ookinetes by recording their position on glass surfaces at 2 or 5 min time intervals (Fig. 6A). The moving velocity of KO parasites was much slower than that of WT (WT = 5.7 ± 1.4 μm/min, KO = 0.6 ± 0.1μm/min, see Fig. 6B). This result indicates that the PbGCβ has a crucial role in ookinete motility.

Development of PbGCβ KO Parasites In Vitro—It appeared that PbGCβ regulates ookinete motility which is necessary for the parasite to traverse the midgut epithelium. In mosquitoes, the parasites must traverse midgut cells and reside on basal lamina to differentiate into oocysts. Thus, we speculated that the KO

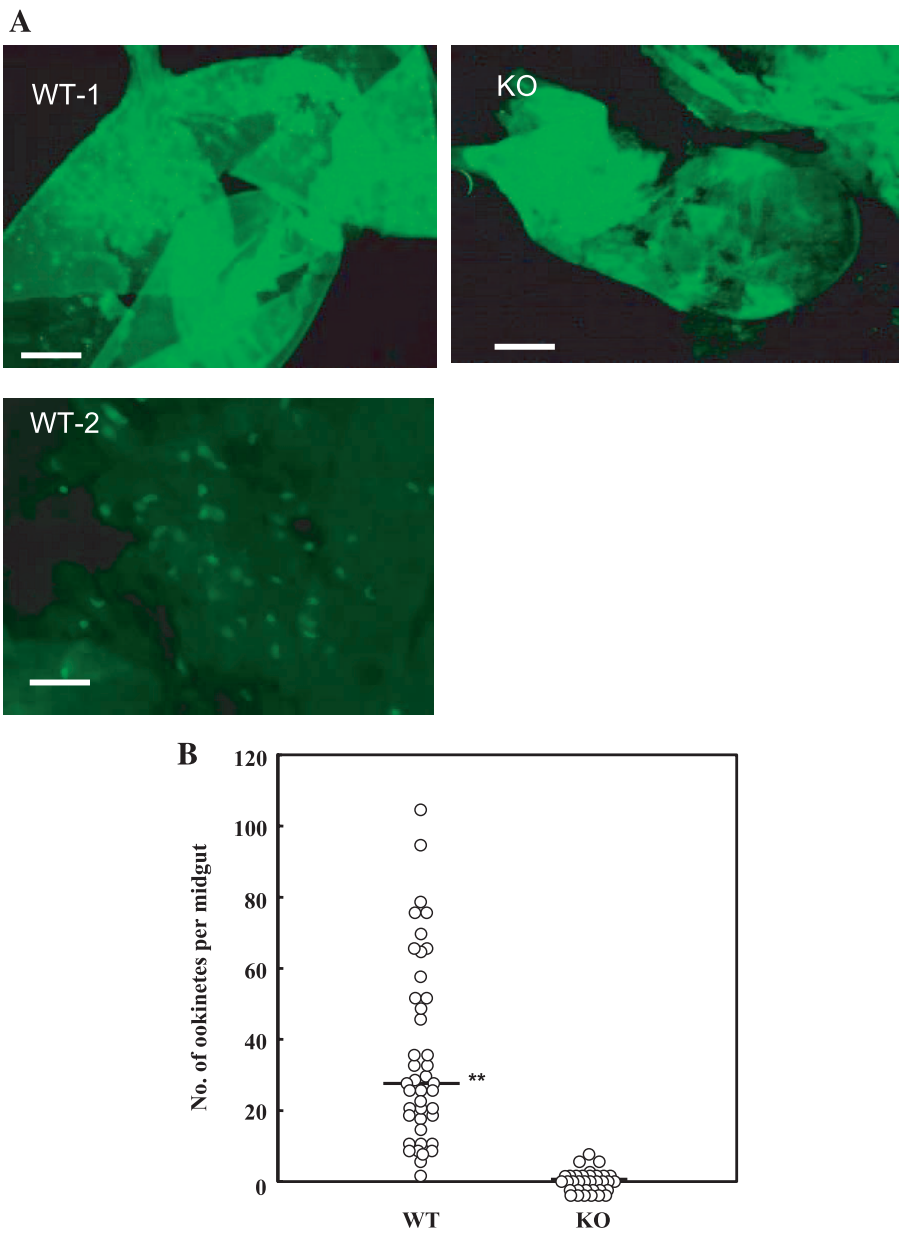

Fig. 4. **Immunofluorescence staining for ookinetes in the midguts with anti-Pbs21 (Green) antibody at 22–24 h post-feeding.** (A) Midguts of the mosquitoes fed on mice infected with WT (WT1 and 2) and PbGCβ KO parasites (KO). Bars in WT1 and KO, and WT2 represent 60 and 20 μm, respectively. (B) The number of ookinetes in the individual midguts fed on WT (left) and KO parasites (right). The number of WT and KO ookinetes per midgut is 36.2 ± 4.0 (N = 42) and 1.2 ± 0.3 (N = 32), respectively. The average is indicated by asterisks. The samples were from three independent preparations.

Table 2. **Localization of ookinetes in mosquito midguts.**[a]

Location of ookinetes	WT (%) (n = 95)	KO (%) (n = 75)
Attached to epithelial cells	9 (9.5)	72 (96.0)
Within epithelial cells	41 (43.2)	1 (1.3)
Close to basement membrane	45 (47.3)	2 (2.7)

[a]Based on examination of ten mosquitoes fed either WT or KO parasites.

ookinetes could not continue their development further since they lost the motility to traverse the midgut epithelium.

As Shown in Table 2, 4% of the KO ookinetes could still migrate to the basal lamina but they did not differentiate into oocysts. This result prompted us to speculate that PbGCβ may have a role in oocyst formation as well. To address this, we assessed the potential of the KO ookinetes for oocyst formation *in vitro*. We cultured KO ookinetes and monitored their development. On day 13 post-culture, the KO parasites transformed into round oocysts and sporozoites (Fig. 7, panel A) and nuclei budded from oocysts into sporozoites (Fig. 7, panel C). Sporozoites containing nuclei was detached from oocysts (Fig. 7, panels D and F). Both oocysts and free sporozoites expressed circumsporozoite protein (CSP), which was verified by using anti-CSP antiserum (Fig. 7, panels B and E). Notably, the KO sporozoites showed motility as WT sporozoites did (data not shown). These results demonstrated that disrupting *PbGC*β gene at least in our culture system, affected ookinete motility but not later development of the parasites.

DISCUSSION

It has been reported that cGMP is important for gametogenesis, especially male exflagellation of *P. falciparum* and

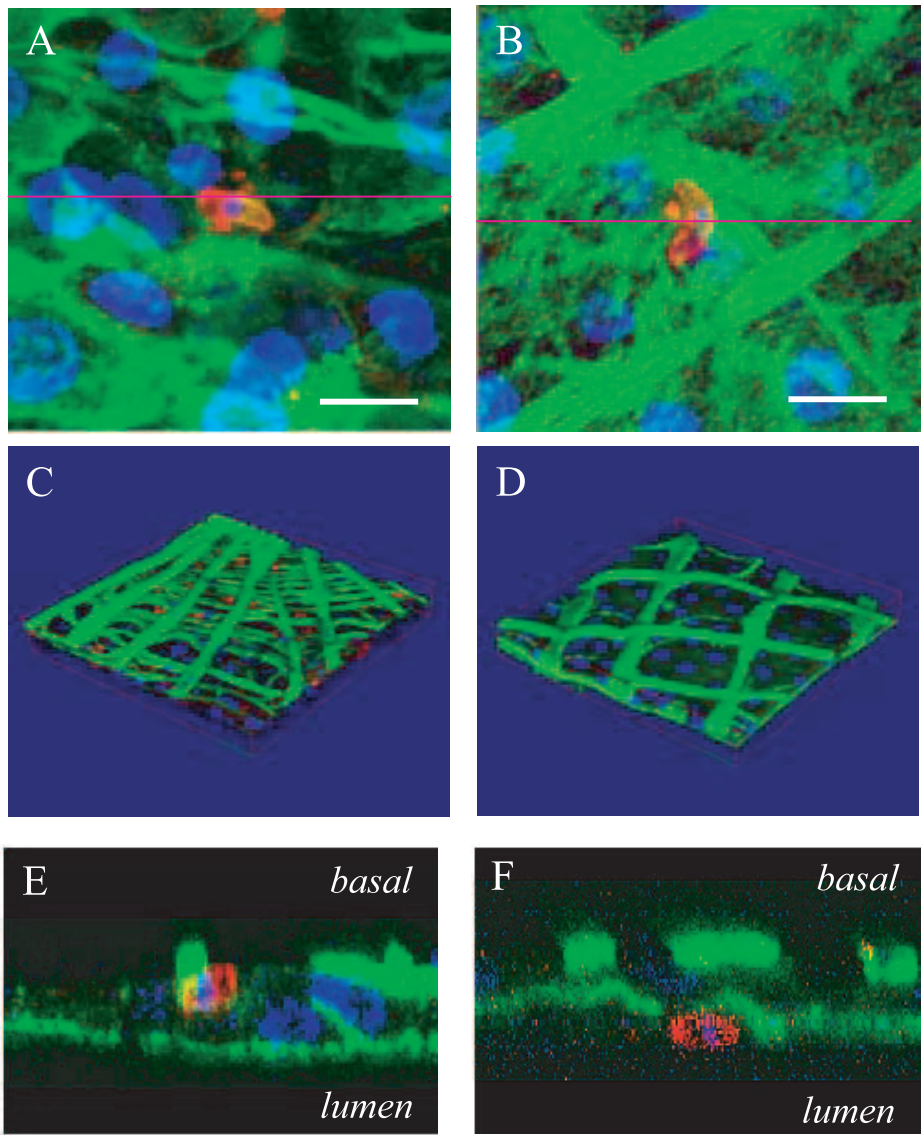

Fig. 5. **Localization of ookinetes in the midguts.** The midguts of the mosquitoes fed on WT (A, C and E) and KO parasites (B, D and F) were stained for ookinetes (red), actin (green) and nuclei (blue) at 24 h post-feeding. A and B; Merged image of WT (A) and KO ookinetes (B). A horizontal section along the pink line, represent a side view as shown in E and F. Bar represents 10 μm. C and D; 3-D image for midguts fed on WT (C) and KO parasites (D). Upper side is the basal side of the midguts which shows thick actin filament. E and F: lateral view of the midgut from mosquitoes fed on WT (E) and KO parasites (F).

P. berghei (*10*, *23*). It has also been reported that gametocytes have membrane associated guanylate cyclase, for which activity is increased by the addition of xanthurenic acid, known as the gametocyte-activation factor (*11*, *12*). A series of evidence imply that gametogenesis triggered by xanthurenic acid is mediated by cGMP signaling. Therefore, we searched for genes encoding membrane-binding guanylate cyclase in the PlasmoDB database and found two genes, PF11_0395 and MAL13P1.301, which are annotated as *PfGC*α and *PfGC*β of *P. falciparum*, respectively (*14*). It has been reported that the both genes are expressed in gametocytes and PfGCβ protein is localized at parasitophorous vacuole (*14*). In addition, the catalytic domains of guanylate cyclase in PfGCβ show guanylate cyclase activity (*14*). It is thus tempting to explore the role of PfGCβ for gametogenesis. Since male exflagellation is an obligatory step for sexual development of all *Plasmodium* species, the molecular machinery behind is assumed to be conserved. We searched for *PfGC*α and *PfGC*β orthologs in *P. berghei*, and found PB001219.00.0 (*PbGC*α), PB000752.03.0 (*PbGC*β) and PB300849.00.0 (*PbGC*β) in the database.

Repeated trials of *PbGC*α gene targeting were unsuccessful, suggesting its essential role in the blood stage parasites. We then tried *PbGC*β targeting and obtained *PbGC*β KO clones. The KO male gametes showed normal exflagellation as compared to WT. Moreover, the KO parasites fertilized, and zygotes differentiated into ookinetes. This suggests that PbGCβ does not have a crucial role in male gametogenesis.

We further investigated the development of the KO parasites and found that they showed severe defect in ookinete motility, which is necessary for the ookinete to traverse midgut epithelium and eventually for successful completion of the life cycle in mosquitoes. All three clones obtained from independent transfection experiments showed the same phenotype, where all clones displayed severe defect in motility and were unable to transform into oocysts. This is the strong evidence that the effect is due to disruption

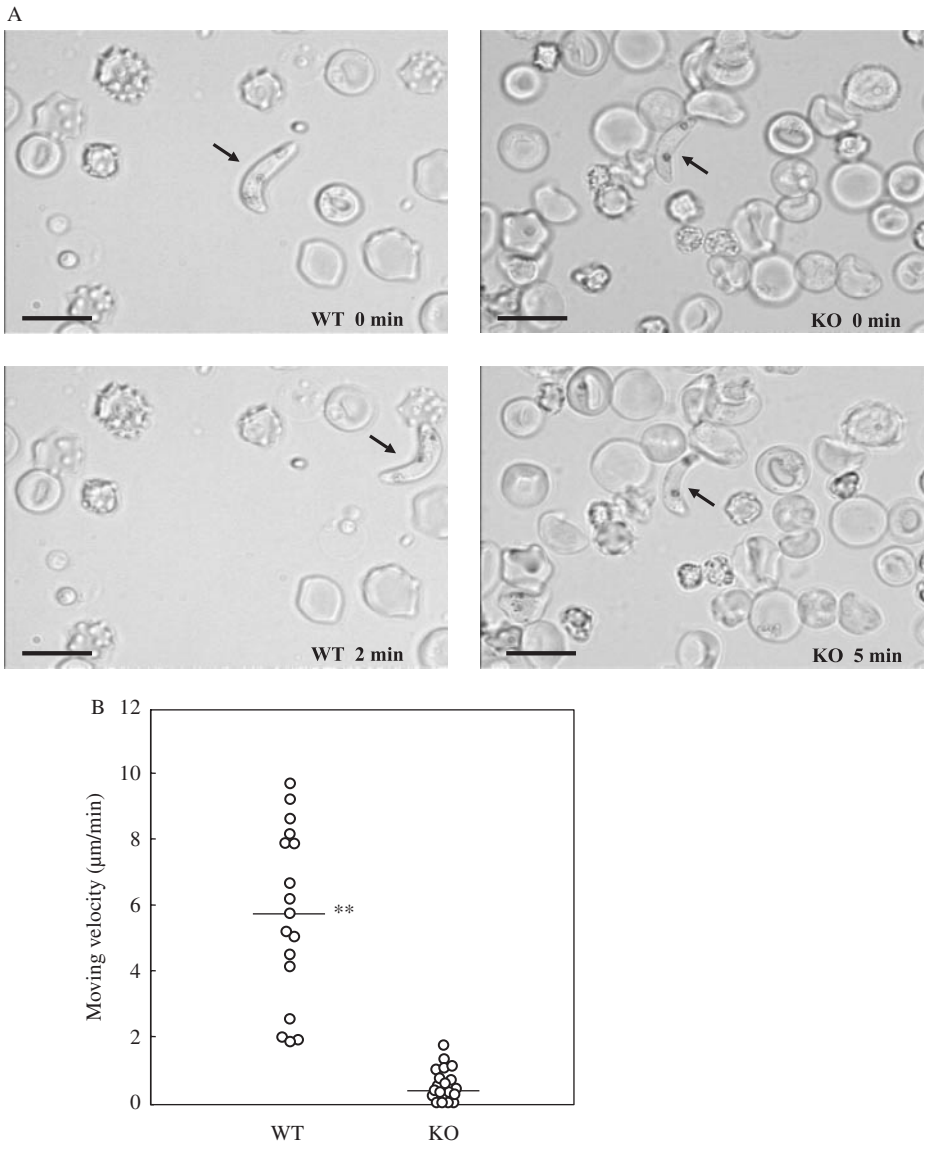

Fig. 6. **Comparison of moving speed between WT and KO ookinetes.** (A) The left side shows photographs of a typical WT ookinete taken 2 min apart, showing displacement. The right shows photographs of a typical KO ookinete taken 5 min apart. Bar represents 10 μm. (B) Velocity of WT and KO ookinetes. The ookinetes were monitored for 5 min by video recorder. The average velocity of WT and KO ookinetes are 5.7 ± 1.4 (N = 57) and 0.6 ± 0.1 μm/min (N = 36), respectively. The average is indicated by asterisks. The samples were obtained from at least four independent preparations.

of the PbGCβ locus, but not because of nonspecific or pleiotropic effects.

At the step where ookinetes migrate into the basal lamina, ookinetes must traverse two barriers; peritrophic membrane (PM) and midgut cells. Disruption of the *PbGC*β gene resulted in severe defect in ookinete motility and the KO ookinetes did not transform into oocysts. Thus, it is conceivable that KO ookinetes may not be able to conquer either of these two barriers. By confocal microscopy, we observed most of the KO ookinetes at microvilli (Fig. 5F), indicating that the KO ookinetes are capable of passing through the PM but arrested at microvilli. Therefore, we conclude that PbGCβ has a role in ookinete motility, which is essential for the parasites to traverse midgut cells.

There are three gene products known to play essential roles in traversing midgut cell membranes. These are: (i) the surface membrane attack ookinete protein (MAOP) in *P. berghei* (*24*), (ii) calcium dependent protein kinase 3 (CDPK3) in *P. berghei* (*25*), and (iii) Circumsporozoite- and TRAP-related protein (CTRP) in *P. berghei* and *P. falciparum* (*16*, *26*, *27*). The MAOP contains membrane-attack complex and perforin (MACPF)-related domain. The MAOP KO ookinetes stop at the point of contact and can not proceed further because of their inability to breach midgut cell membranes (*24*). Since PbGCβ does not contain MACPF or related domains, it is unlikely that PbGCβ has the membrane breaching activity. Rather, as shown in Fig. 6, the moving velocity of the PbGCβ KO ookinetes is much slower than that of WT, indicating that PbGCβ is involved in the ookinete motility. It is reported that CDPK3 KO ookinetes can migrate on the surface of the matrigel (horizontal locomotion) but can not invade into the gel (vertical locomotion), indicating that CDPK3 regulates vertical locomotion (*25*). We measured the moving velocity of the ookinetes by recording the moving distance of ookinetes on the slide glass at time interval. As the moving velocity of PbGCβ KO ookinetes is much slower than that of WT, PbGCβ may regulate the motility affecting the horizontal locomotion. Whether PbGCβ affects vertical locomotion as well, further analysis

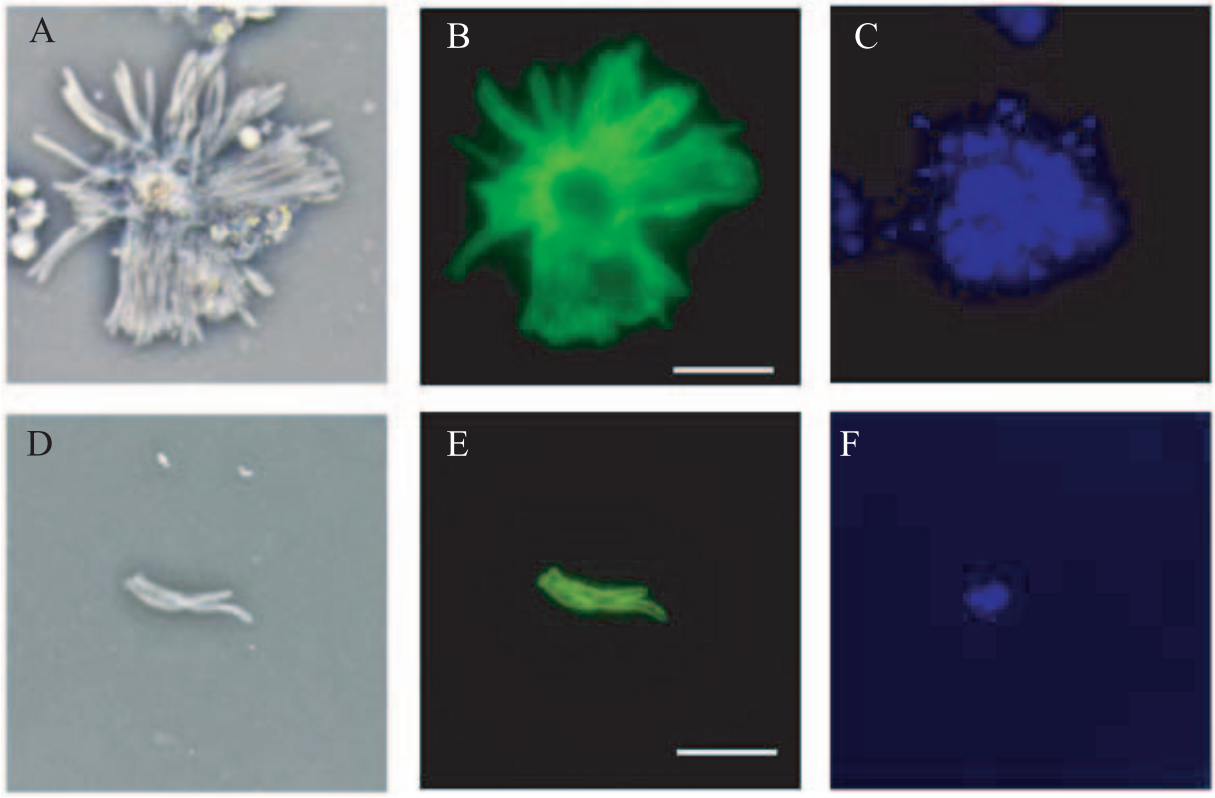

Fig. 7. **KO ookinetes differentiated into oocysts and sporozoites *in vitro***. KO ookinetes were cultured *in vitro* for 13 days. The KO ookinetes differentiated into oocysts (panels A to C) and the free sporozoites detached from oocysts (panels D–F). The oocysts and sporozoites were observed under bright field (panels A and D) and stained by anti-CSP antibody (panels B and E) and DAPI (panel C and F). Bar represents 10 μm.

is required. The CTRP KO ookinetes showed severe defect in motility and lost the ability to traverse midgut epithelium. However, the CTRP KO ookinetes displayed bending and straightening (*16*). In contrast to this, the PbGCβ KO ookinete does not show such motility. Taken together, although all these KO ookinetes fails to invade midgut cells, the detailed comparison of the phenotypes of PbGCβ KO ookinetes with those of MAOP, CDPK3 and CTRP KO ookinetes suggest that each molecule has distinct function in ookinete motility.

Although 4% of the KO ookinetes could enter into the basal lamina, they failed to transform into oocysts (Table 2). This implies that PbGCβ could have a role in oocyst formation as well. To test this hypothesis, we cultured the KO ookinetes and found that the KO ookinetes differentiated into oocysts and formed sporozoites, suggesting that PbGCβ is not essential for sporozoites formation. Moreover, *in vitro* generated sporozoites display normal locomotion (data not shown). Taken together, PbGCβ exerts its function in a stage specific manner, whereby PbGCβ regulates ookinete motility but does not affect further development of the parasites. Mosquitoes mount potent immune responses upon parasite infection, which is directly linked to parasite mortality. It has been estimated that of the 10,000 gametocytes that enter the midgut, nearly 1,000 ookinetes develop. Of these, less than 5 develop into oocysts in malaria parasite–susceptible mosquitoes (*1*, *28*, *29*). Thus, a small number of KO ookinetes on basal lamina may be eliminated by the host immune system.

Molecular motor machineries used in parasite gliding motility are highly conserved among apicomplexan parasites (*30*). In *Plasmodium* spp., *T. gondii*, *Eimeria tenella* and *Cryptosporidium parvum*, it is known that members of the thrombospondin-related anonymous protein (TRAP) family are secreted on the parasite's surface from organelles called micronames, rhoptories and dense granules. During the forward movement of parasites, the adhesive molecules are transported to the posterior of the cell, cleaved and released from the cells, thereby creating forward locomotion of parasites (*31–33*). TRAP family proteins are linked internally to an anchor that is attached to a series of membrane structures, underlying the outer plasma membrane of the parasites, called the inner membrane complex (IMC). Between the plasma membrane and IMC lies an internal actin-myosin motor that drives cell motility (*34*). The same molecular components are also used for host cell invasion. The remaining question is how PbGCβ exerts its function in motility. Is the P-type ATPase-like domain or the guanylate cyclase domain or both necessary for ookinete motility? It has been shown that DKTGT(L/I)T)C, which is located in the cytosolic domain, is an invariant signature in all P-type ATPases. The aspartate must be phosphorylated for ion transporting activity (*35*). This motif is not conserved in PbGCβ. Other conserved domains, GDGXND in the hinge domain and

(TSND)GE(SNT) in the transduction domain are not present in PbGCβ. These decisive deviations of the PbGCβ sequence from the ATPase consensus imply that P-type ATPase-like domain of PbGCβ has another unknown function. Carucci *et al.* (*14*) reported that the recombinant protein of the P-type ATPase domain of PfGCβ did not show ATPase activity while guanylate cyclase activity was confirmed in the corresponding domain of PfGCβ. Identities of the catalytic domains C1a and C2a in the guanylate cyclase domain of PbGCβ with corresponding domains of PfGCβ are 84% and 89%, respectively, suggesting that PbGCβ encodes functional guanylate cyclase. It is likely that cGMP generated by PbGCβ could activate downstream signaling and eventually drive parasite motility. cGMP transduces its signal *via* a cGMP-dependent protein kinase (PKG), a cGMP-gated ion channel and a cGMP phosphodiesterase (PDE). It has been reported that compound 1 (the trisubstituted pyrrole 4-[2-(4-fluorophenyl)-5-(1-methylpiperidine-4-yl)-1*H*-pyrrol-3-yl]) inhibits invasion and gliding motility of *T. gondii* tachyzoite and *E. tenella* sporozoites by interrupting the discharge of adhesive protein. Moreover, the target molecule of compound 1 is found to be PKG (*36*). In this context, it is plausible that the guanylate cyclase domain in PbGCβ may have this function. The validation of the guanylate cyclase activity in PbGCβ and the determination of the regulatory domain in PbGCβ for ookinete motility are underway. This functional study may provide novel strategy to prevent malaria parasite development in mosquitoes aiming at the parasite motility.

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