A member of the CPW-WPC protein family is expressed in and localized to the surface of developing ookinetes

Niwat Kangwanrangsan1,5, Mayumi Tachibana1,2, Rachaneeporn Jenwithisuk1,6, Takaumi Tsuboi3, Suda Riengrojpitak4, Motom Torii1,2 and Tomoko Ishino1,2*

Abstract

**Background:** Despite the development of malaria control programs, billions of people are still at risk for this infectious disease. Recently, the idea of the transmission-blocking vaccine, which works by interrupting the infection of mosquitoes by parasites, has gained attention as a promising strategy for malaria control and eradication. To date, a limited number of surface proteins have been identified in mosquito-stage parasites and investigated as potential targets for transmission-blocking vaccines. Therefore, for the development of effective transmission-blocking strategies in epidemic areas, it is necessary to identify novel zygote/ookinete surface proteins as candidate antigens.

**Methods:** Since the expression of many zygote/ookinete proteins is regulated post-transcriptionally, proteins that are regulated by well-known translational mediators were focused. Through in silico screening, CPW-WPC family proteins were selected as potential zygote/ookinete surface proteins. All experiments were performed in the rodent malaria parasite, *Plasmodium yoelii* XNL. mRNA and protein expression profiles were examined by RT-PCR and western blotting, respectively, over the course of the life cycle of the malaria parasite. Protein function was also investigated by the generation of gene-disrupted transgenic parasites.

**Results:** The CPW-WPC protein family, named after the unique WxC repeat domains, is highly conserved among *Plasmodium* species. It is revealed that CPW-WPC mRNA transcripts are transcribed post-transcriptionally, proteins that are regulated by well-known translational mediators were focused. Through in silico screening, CPW-WPC family proteins were selected as potential zygote/ookinete surface proteins. All experiments were performed in the rodent malaria parasite, *Plasmodium yoelii* XNL. mRNA and protein expression profiles were examined by RT-PCR and western blotting, respectively, over the course of the life cycle of the malaria parasite. Protein function was also investigated by the generation of gene-disrupted transgenic parasites.

**Conclusions:** It is demonstrated that PyCPW-WPC-1 can be classified as a novel, post-transcriptionally regulated zygote/ookinete surface protein. Additional studies are required to determine whether all CPW-WPC family members are also present on the ookinete surface and share similar biological roles during mosquito-stage parasite development. Further investigations of CPW-WPC family proteins may facilitate understanding of parasite biology in the mosquito stage and development of transmission-blocking vaccines.

**Keywords:** Malaria, Transmission-blocking vaccine, Mosquito, Post-transcriptional regulation, CPW-WPC protein

* Correspondence: tishino@m.ehime-u.ac.jp
1Department of Molecular Parasitology, Graduate School of Medicine, Ehime University, Shitsukawa, Toon, Matsuyama, Ehime 791-0295, Japan
2Division of Molecular Parasitology, Proteo-Science Center, Ehime University, Shitsukawa, Toon, Matsuyama, Ehime 791-0295, Japan
Full list of author information is available at the end of the article

© 2013 Kangwanrangsan et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Background
Malaria is a serious tropical disease caused by infection with *Plasmodium* parasites that are transmitted via *Anopheles* mosquitoes. While intense efforts in malaria control have affected a gradual decrease in the global number of malaria cases and deaths every year since 2005, an estimated 3.3 billion people were still at risk of malaria infection in 2010 [1]. The World Health Organization (WHO) has recommended two major strategies currently in use for malaria control: the prevention of mosquito bites using long-lasting insecticide-treated mosquito nets (LLINs) and indoor residual spraying (IRS) and the management of malaria cases with artemisinin-based combination therapy (ACT) and rapid diagnosis [1,2]. Despite extensive attempts to develop vaccine strategies to inhibit parasite proliferation in human hosts (pre-erythrocytic- and erythrocytic-stage vaccines), no vaccines have been developed for clinical use to date [3]. In contrast, transmission-blocking vaccine (TBV) strategies, which aim to interrupt parasite development in the mosquito vector, have recently been revealed as effective methods for the elimination and eradication of malaria [4]. Moreover, it has been suggested that TBV, in combination with blood-stage vaccines or anti-malarial drugs, could prevent the emergence of vaccine- or drug-resistant parasites [5,6].

Malaria transmission to mosquito vectors is initiated when male and female gametocytes are acquired during blood sucking. Gametocytes undergo development into gametes in the midgut and then mate to form zygotes. Later, they mature into ookinetes, the invasive form of the parasite that can penetrate the midgut epithelium. The principle idea of TBV is to interrupt parasite mating, development, or invasion inside the mosquito midgut using antibodies produced in human hosts and acquired, together with gametocytes, by mosquitoes during the blood meal. Therefore, TBV has the potential to break the life cycle of parasites in mosquito vectors [7].

Screening for TBV target antigens typically focuses on proteins expressed on the surface of parasites during the sexual stages (gametocytes and gametes) and/or mosquito stages (zygotes and ookinetes) [8,9]. Several surface proteins have been identified as candidates for TBV antigens, including P25, P28, P230, P48/45, and PCCps/LAPs (LCCL/lectin adhesive-like protein) [10,11]. Studies using membrane-feeding assays have demonstrated that anti-P25 and anti-P28 antibodies inhibit parasite invasion in the mosquito midgut [12]. However, stepwise clinical examinations, together with further screening for candidate antigens, are necessary for the development of TBV strategies.

To screen candidate antigens for TBV, two criteria were set: 1) proteins should be expressed exclusively from gametes to ookinetes, and 2) proteins should be localized to the surface of parasites. Progress in genetic manipulation has revealed a number of proteins that are important for parasite development in mosquitoes, such as calcium-dependent protein kinase 4 (CDPK4) in exflagellation [13], P48/45 and hapless 2/generative cell-specific 1 (HAP2/GCS1) in gamete fertilization [14-16], and several proteins in ookinete motility and invasion (reviewed in [17]). Most of these molecules are regulated post-transcriptionally by messenger ribonucleoproteins, development of zygote inhibited (DOZI), and homolog of worm CAR-1 and fly Trailer Hitch (CITH) [18,19].

In the current study, a novel malaria protein family, CPW-WPC, which is expressed on the surface of developing ookinetes, is identified by focusing on predicted secretory and post-transcriptionally regulated proteins. These findings suggest that CPW-WPC family proteins may be potential candidate antigens for transmission-blocking strategies.

Methods
Experimental infection of mice by parasites
All the animal experiments were conducted in accordance with the guide for animal experimentation at Ehime University, Graduate School of Medicine. Eight-week-old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan), pretreated with 0.2 mL of 0.6 mg/mL phenylhydrazine, were injected intraperitoneally with *P. yoelii* 17XL parasites. To obtain only gametocytes, asexual-stage parasites were eliminated by intraperitoneal injection of 0.25 mL sulfadiazine (2 mg/mL) 24 h before blood collection.

Collection of parasites
For blood-stage parasites, infected blood (at 10–20% parasitaemia) was mixed with 5 volumes of ice-cold incomplete medium (ICM; RPMI1640 medium, pH 7.4, Invitrogen) and then passed through a sterile CF11 filter (Whatman, England) to remove white blood cells. Cells were pelleted at 1,200 × g for 5 min and washed with ice-cold ICM before antigen preparation. For *in vitro* ookinete culture, infected blood was immediately mixed with 5 volumes of SA buffer (10 mM Tris, 150 mM NaCl, 10 mM glucose, pH 7.3, warmed to 37°C) to prevent gamete exflagellation and then passed through a sterile CF11 filter. After washing with ICM (warmed to 37°C), the parasites were resuspended with 20 volumes of ookinete culture medium (OCM; 24°C), composed of RPMI1640 medium containing 20% heat-inactivated fetal calf serum, 0.367 mM hypoxanthine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 5 U/mL heparin (pH 8.3). The parasites were then transferred into culture flasks and incubated at 24°C. At 0, 1, 4, 16, and 24 h after incubation, equal volumes of cultured ookinetes were collected and washed with ice-cold ICM before antigen preparation.
Preparation of parasite antigens
Parasite samples were purified by density gradient centrifugation using Percoll (GE Healthcare, USA). Briefly, parasite pellets were gently mixed with 4 volumes of ICM, layered onto 50% Percoll, and centrifuged at 410 x g for 20 min at room temperature. Purified parasites were collected from the interface, washed twice, and resuspended in 2 volumes of ICM. The purity was checked microscopically using Giemsa-stained blood smears, and the parasite number was determined by counting under a haemocytometer before spotting onto glass slides for the immunofluorescent assay. To prepare the antigens for western blotting, the purified parasites were treated for 10 min at 4°C with 0.15% saponin in ICM containing protease inhibitors (Roche, Germany). The samples were washed twice with 0.1 M phosphate-buffered saline (PBS) containing protease inhibitors (PBS-PI) and were stored as pellets at −80°C until use. For the collection of sporozoites, the salivary glands were dissected from infected mosquitoes at day 15 post-feeding and then ground in ice-cold ICM to release sporozoites.

Bioinformatics
The genomic sequences used in this study were obtained from PlasmoDB, the Plasmodium Genomics Resource [20]. The putative signal peptide and CPW-WPC domains were also investigated. Multiple sequence alignments of CPW-WPC family members were performed with ClustalW (MegAlign; Lasergene®).

RT-PCR of pycpw-wpc-1 and CPW-WPC family member transcripts
Total RNA was isolated from in vitro cultured oocokites using the RNeasy Micro Kit (Qiagen). To obtain cDNA, total RNA was subjected to reverse transcription and subsequent PCR using the PrimeScript reagent kit (TAKARA, Japan). The transcriptional profiles of pycpw-wpc-1, pyp00599, pyp03515, pyp04297, and py1isp70 were determined by conventional PCR at 28 cycles using gene-specific primers (see Additional file 1). cDNA concentrations were normalized across samples to pyhsp70 expression. PCR products were analysed using ImageQuant LAS 4000 (GE Healthcare), and band intensities were measured using Adobe Photoshop (Adobe System Inc., USA).

Expression of recombinant PyCPW-WPC-1 (rPyCPW-WPC-1)
The target gene was amplified from P. yoelii 17XNL zygote-enriched cDNA by PCR using the Phusion High-Fidelity DNA Polymerase (FINNZYMES, Finland). A gene-specific primer set (forward, 5’-CTCGAGAAAAACTTTTGCTCTTTCTGGAGACG-3’ and reverse, 5’-GATCCCTTAAATATGTACGGTATTTGAATCC-3’) was designed to amplify the target sequence (1,539 base pairs), excluding the N-terminal signal sequence. The PCR fragment was cloned into the pEU-E01-GST-TEV-MCS-N2 vector (CellFree Sciences Co. Ltd., Matsuyama, Japan), as previously described [21]. Plasmids were then utilized for protein production in a wheat germ cell-free protein expression system using the bilayer translation reaction method [22,23]. GST-fused rPyCPW-WPC-1 was bound to a glutathione Sepharose 4B column, followed by cleavage with a tobacco etch virus protease to elute the rPyCPW-WPC-1 protein from the column. The yield was analyzed by Bradford’s assay and conventional SDS-PAGE with Coomassie brilliant blue staining.

Production of anti-PyCPW-WPC-1 antibodies
Eight-week-old female BALB/c mice were immunized by intraperitoneal injection of rPyCPW-WPC-1 in Freund’s adjuvant (Wako, Japan) 3 times (30 μg in 0.2 mL each) at 3-week-intervals. Immune sera were obtained two weeks after the last immunization. The reactivity and specificity of anti-rPyCPW-WPC-1 antisera were determined by ELISA and western blotting.

SDS-PAGE and western blotting
Parasite pellets were extracted with 1% Triton X-100 in PBS-PI. To solubilize the parasite antigens, the mixture was incubated on ice for 1 h. After centrifugation, the supernatant was recovered into a new microcentrifuge tube and mixed with loading buffer containing 2-mercaptoethanol at 4% of the final volume. Antigens were then loaded onto 12.5% polyacrylamide gels (ATTO Bioscience & Biotechnology, Japan) at 0.5–1 × 10⁵ parasites/lane and run in an electrophoresis buffer under a constant current. The separated proteins were then transferred onto PVDF membranes (Millipore) using a semidry transfer system. After transfer, membranes were incubated with 5% skim milk in PBS containing 0.1% Tween20 (PBST) at room temperature for 2 h, followed by incubation with mouse anti-PyCPW-WPC-1 antiserum (dilution, 1:100) for 1 h. After washing with PBST, the membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (dilution, 1:20,000; Invitrogen) for 30 min. Unbound antibodies were washed out with PBST, and membranes were incubated with a chemiluminescent-HRP substrate (Immobilon Western, Millipore), wrapped in a cassette, and exposed to X-ray film (Fujifilm Corporation, Tokyo, Japan). Exposed films were developed using a CEPROS SV machine (Fujifilm Corporation).

Indirect immunofluorescent assay (IFA)
Antigen slides were fixed with ice-cold acetone for 5 min. Nonspecific binding was then blocked with 5% skin milk in PBS at 37°C for 30 min before incubation with antibodies (mouse anti-PyCPW-WPC-1 at a dilution of 1:100 and rabbit anti-Pys25 at a dilution of...
1:10,000) at 37°C for 1 h. The slides were then washed in PBS for 5 min and subsequently incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen) diluted in PBS containing 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) at 37°C for 30 min. After washing out the unbound antibodies, the coverslips were mounted with Prolong Gold antifade reagent (Invitrogen), and the slides were observed under a confocal microscope (LSM710; Carl Zeiss, Germany). The resulting images were analysed using the Zen software (Carl Zeiss, Germany).

Targeted gene disruption of pycpw-wpc-1

Pycpw-wpc-1 was disrupted by double-crossover homologous recombination in *P. yoelii* 17XLN. Two basic plasmids, pPbDT3UB12 and pHDEF1-mh-R12, were used for the construction of transfection plasmids, as previously described [24]. In brief, target sequences were amplified by PCR using KOD Plus DNA polymerase (Toyobo, Japan) with primer sets targeting the 809-base pair region upstream (–forward, 5′-CTCGAGATATGCATAATTGTG AATAGTTATTGG-3′ and –reverse, 5′-GGATCCGACCC CTATAATAAAGGTCTGTC-3′) and 755-base pair

![Figure 1](http://www.malariajournal.com/content/12/1/129)

**Figure 1** Schematic representation of *Plasmodium* CPW-WPC family proteins. (A) Diagram showing the common structure shared between CPW-WPC family proteins. Each protein contains a signal peptide and 5 repeated CPW-WPC domains, each having 4–6 cysteine residues. (B) ClustalW alignment of the first 2 CPW-WPC domains showing conserved amino acids (asterisks) and the arrangement of cysteines (highlight). Note that the WxC pattern is typically located at the end of each domain (box).
region downstream (−forward, 5′-AGATCTACCGAGATGATTATAATGGACCTTGTC-3′ and −reverse, 5′-CTCGAGCCGATGTATTGAGACGTCTTGACATGTG-3′) of pycpw-wpc-1. PCR products were subsequently digested and inserted into pHDEF/Ub12. Using Gateway Technology (Invitrogen), the modified pHDEF/Ub12 plasmid was then subjected to a BP recombination reaction with the donor vector pDONR221 to give the entry plasmid. This entry plasmid was then subjected to an LR recombination reaction with pHDEF-1-mb-R12 to yield the final construct, which was linearized with XhoI before use. Enriched schizonts were transfected with 20 μg of linearized construct by electroporation using Nucleofector (Lonza) with a human T cell solution and the U-33 program. Transfected parasites were delivered into 8-week-old BALB/c female mice by intravenous injection. Twenty-four hours later, the mice were treated with pyrimethamine (70 μg/mL) via their drinking water. Infected blood was collected to examine the integration of the disruption cassette by PCR using the KOD FX DNA polymerase with a unique set of primers. Successful transfected parasites were cloned using the limiting dilution technique, and cloned parasites were cultured for oocystes. The expression of PyCPW-WPC-1 was measured by western blotting and IFA using mouse anti-PyCPW-WPC-1 antibodies.

Mosquito feeding assay
More than 50 female Anopheles stephensi mosquitoes were allowed to feed on mice infected with wild-type- or gene-disrupted parasites, and only mosquitoes that showed complete engulfment of the blood were selected for an additional two weeks of incubation at 24°C. For infectivity analysis, mosquito midguts were dissected on day 10 post-feeding, and oocyste numbers were counted under a microscope. To examine sporozoite-formation ability, sporozoites were collected from midguts on day 15 post-feeding and counted.

Table 1 Members of CPW-WPC domain protein family

| # | PBANKA (PY ortholog) | gDNA coding seq. amino acid residues | MW (kDa) | pl | SP | TM/GPI | CPW-WPC domain | DOZI KO vs. WT (confidence) |
|---|----------------------|-----------------------------------|----------|----|----|--------|----------------|---------------------------|
| 1 | 135250 (PyCPW-WPC-1) | 2,769, 1,623, 540 | 63.11 | 4.95 | + | - | 5 | -2.71 (0.75) |
| 2 | 134630 (PY04297) | 1,407, 1,407, 468 | 55.01 | 7.04 | + | - | 5 | -3.66 (0.72) |
| 3 | 121830 (PY00599) | 1,584, 1,584, 527 | 62.13 | 6.83 | + | - | 5 | -2.22 (0.75) |
| 4 | 101540 (PY00690) | 2,843, 1,116, 371 | 42.99 | 6.69 | + | - | 5 | -2.26 (0.73) |
| 5 | 094340 (PY00905) | 3,584, 1,752, 583 | 67.10 | 6.79 | + | - | 5 | -2.06 (0.75) |
| 6 | 144930 (PY03515) | 1,786, 1,644, 547 | 63.33 | 8.48 | - | - | 5 | -2.38 (0.73) |
| 7 | 124520 (PY07114) | 2,196, 753, 250 | 28.55 | 4.79 | + | - | 2 | -2.46 (0.72) |
| 8 | 112880 (PY04202) | 1,958, 840, 279 | 32.29 | 4.31 | + | - | 2 | -2.51 (0.72) |

Data from PlasmoDB (http://plasmodb.org/plasmo)

Results
Identification of Plasmodium CPW-WPC family members
To identify novel mosquito-stage surface proteins, post-transcriptionally regulated proteins were explored since many zygote/ookinete proteins have been reported to be regulated post-transcriptionally. In a previous study, Mair et al. showed that the mRNA levels of 117 genes were dependent on both DOZI and CITH, which are well-studied messenger ribonucleoproteins expressed in gametocytes [19]. It was founded that seven of these 117 proteins belonged to the CPW-WPC family, which is named after the unique WxC motif found at the end of repeated domains. Amino acid sequence analyses showed that CPW-WPC family proteins are conserved among Plasmodium species and contain an N-terminal signal peptide. In silico analysis using PlasmoDB [25] demonstrated that eight CPW-WPC proteins share a similar structure, including a signal peptide and five repeated CPW-WPC domains with four to six cysteine residues (Figure 1A and Table 1). The sequence alignment of the first two domains is shown in Figure 1B. Moreover, CPW-WPC family proteins were also found in other members of Apicomplexans, such as Theileria parva (TP04_0816, TP04_0805, TP04_0558, and TP04_0183), Babesia bovis (BBOV_111009100, BBOV_111000280, BBOV_11004430, and BBOV_11004280), and Toxoplasma gondii (TGM E49_066770, TGME49_047310, and TGME49_006440).

Here, one of the CPW-WPC family proteins (PBANKA_135250) was selected for further investigation in another rodent malaria parasite (P. yoelii 17XNL). Since the nucleotide sequence of the orthologous gene in P. yoelii (designated as PyCPW-WPC-1) has not yet been fully identified on PlasmoDB, PCR analyses of both genomic DNA and cDNA were performed to determine the primary sequence and its exon-intron structure. Multiple amino acid sequence alignment of the PyCPW-WPC-1 protein showed 93–94% and 50–55% identity among the orthologs related to rodent (PBANKA_135250 and PCHAS_135710) and human malaria parasites (PF3D7_
Furthermore, all eight CPW-WPC family members are described on PlasmoDB as DOZI-dependent post-transcriptionally regulated proteins. Taken together, these data suggest that CPW-WPC domain proteins tend to be expressed in zygotes and/or ookinetes.

Expression of \textit{pycpw-wpc-1} mRNAs before ookinete formation

RT-PCR analyses were performed to investigate the expression profiles of \textit{pycpw-wpc} family genes during mating and ookinete development \textit{in vitro}. As shown in Figure 2A, \textit{pycpw-wpc-1} transcripts were detected from 0–1 h of ookinete culture, which included gametocytes, gametes, and early zygotes. After zygote formation, the \textit{pycpw-wpc-1} expression was reduced significantly (4 h) and was barely detectable after 16 and 24 h of ookinete culture (Figure 2A). Other family members (\textit{py00599}, \textit{py03515}, and \textit{py04297}, corresponding to PBANKA\_121830, PBANKA\_144930, and PBANKA\_134630, respectively) showed similar transcription patterns, i.e., highly expressed until zygote formation, with a subsequent decrease in expression. Among the examined molecules, \textit{py04297}, which exhibited the highest expression, had the same expression pattern as \textit{pycpw-wpc-1}, suggesting that these proteins may have coordinate functions (Figure 2B). In contrast, the expression of \textit{py00599} and \textit{py03515} peaked at 1 h of ookinete culture. These results reveal that \textit{pycpw-wpc} family genes are expressed predominantly before zygote formation and that the expression of these targets decreases during the course of ookinete development.

![Figure 2](http://www.malariajournal.com/content/12/1/129)
PyCPW-WPC-1 is a zygote/ookinete stage-specific protein.

To obtain specific antibodies targeting PyCPW-WPC-1, full-length recombinant PyCPW-WPC-1 without a signal sequence (rPyCPW-WPC-1) was synthesized using a wheat germ cell-free protein expression system. After purification, rPyCPW-WPC-1 was obtained as the major band with a molecular weight of about 60 kDa (Figure 2C), and this purified product was then used for immunization in mice. Antisera were collected from immunized mice and used for western blotting to study the expression of the target protein during the malaria life cycle, including schizont-enriched blood-stage parasites, ookinetes, and sporozoites collected from mosquito salivary glands. The results demonstrated that PyCPW-WPC-1 was produced exclusively in ookinetes (Figure 2D). Moreover, western blotting analysis using the same in vitro cultured ookinete samples as used for transcriptional analysis (see Figure 2A) showed that PyCPW-WPC-1 expression was very low in gametes (0 h), despite the presence of pycpw-wpc-1 transcripts. The expression of PyCPW-WPC-1 protein became prominent at 1 and 4 h of ookinete culture and decreased gradually until 24 h of ookinete maturation (Figure 2E).

From morphological observations using Giemsa’s stain, it is concluded that translation of PyCPW-WPC-1 starts during zygote and early ookinete development. Together with our transcriptional data, these results demonstrate that the expression of PyCPW-WPC-1 is regulated post-transcriptionally, as predicted by microarray analyses of pbdozi-disrupted parasites [18].

Localization of PyCPW-WPC-1 to the zygote/ookinete surface

Next, the localization of PyCPW-WPC-1 during mosquito-stage parasite development, from gametocytes to ookinetes,
was investigated. Immunofluorescent staining using mouse anti-PyCPW-WPC-1 antiserum revealed a strong signal on the surface of zygotes, retorts, and mature ookinetes, but not on gametocytes (Figure 3). The staining pattern was similar to that of Pys25, a well-known ookinete surface protein. In addition, another family member, Py03515, also showed a similar surface localization pattern (shown in Additional file 2).

PyCPW-WPC-1 is not essential for ookinete formation
To investigate the function of PyCPW-WPC-1, targeted gene disruption by homologous recombination was performed (Figure 4A). Gene-disrupted parasites were selected by administration of antimalarial drugs to infected mice, and after cloning, disruption of the pycpw-wpc-1 locus was confirmed by PCR using specific primers. To examine the formation of ookinetes by mutant parasites,
in vitro ookinete cultures were grown. *pycw-wpc-1*−/− ookinetes appeared to have a normal morphology (see Figure 4C), with an almost equivalent efficiency to the formation of wild-type ookinetes, indicating that PyCPW-WPC-1 is not essential for ookinet development in vitro. Using these cultured ookinetes, depletion of PyCPW-WPC-1 was confirmed by western blotting (Figure 4B) and immunofluorescent analyses (Figure 4C). The transmission ability of *pycw-wpc-1*-disrupted parasites was analysed by mosquito feeding assay. As shown in Figure 4D, the oocyst number of *pycw-wpc-1*−/− parasites was not significantly different from that of wild-type parasites examined by unpaired t-tests, indicating that CPW-WPC-1 is dispensable during oocyst formation in vivo. Furthermore, to examine the efficiency of sporozoites production, the numbers of sporozoites collected from midguts at day 15 post-feeding were compared. The average number of *pycw-wpc-1*−/− sporozoites (19,783 ± 11,176) was equivalent to that of wild-type sporozoites (18,057 ± 4,977), strongly suggesting that CPW-WPC-1 is not essential for parasite transmission to mosquitoes. On the other hand, since at least two members of the CPW-WPC protein family are co-expressed and colocalized on the ookinete surface, it is possible that other family members could compensate for the function of PyCPW-WPC-1 during ookinete development.

**Discussion**

After the discovery that the zygote/ookinete surface proteins, P25 and P28, could be good candidate antigens for TBVs, considerable effort has been made to identify additional surface proteins; however, these attempts have not been successful. This study demonstrates that a member of the CPW-WPC protein family (PyCPW-WPC-1) is expressed during zygote and ookinete development in mosquitoes and is localized to the surface of developing ookinetes in *P. yoelii*. Database analyses using *P. berghei* demonstrate that the CPW-WPC family contains eight proteins, as shown in Table 1. Interestingly, studies have shown that the transcription of all CPW-WPC members is reduced in *pbdzo1* mutants [18], indicating that all family members are expressed during the sexual stage under DOZI-dependent post-translational control. This characteristic can also be found in several hundred other proteins expressed in ookinetes, as demonstrated by studies on regulatory molecules, such as DOZI, CITH, HMGB2, and MISFIT [18,19,26,27].

Similar to the structure of P25 and P28, CPW-WPC proteins also contain conserved cysteine domains (Figure 1A). Despite the absence of transmembrane or glycosylphosphatidylinositol (GPI) anchor domains, two members of the CPW-WPC protein family were found to be localized to the ookinete surface (Figure 3). Moreover, by algorithmic analysis using MAAP [28], CPW-WPC proteins appeared in the predicted adhesin group, suggesting that they may be involved in host parasite interactions during ookinete development and/or migration in the mosquito midgut. It was also shown here that targeted *pycw-wpc-1* gene disruption had no significant effect on ookinete or sporozoite formation. Since at least one other CPW-WPC protein (Py03515) showed a similar ookinete surface localization pattern (Additional file 2), it is possible that other family members could compensate for PyCPW-WPC-1 function. Therefore, it would be interesting to determine whether all CPW-WPC family members are expressed at the same time on the ookinete surface or whether there is exclusive expression of these proteins, as is seen for *var* genes, a family of *Plasmodium falciparum* virulence genes [29]. Thus, for further functional analysis of this protein family, double gene disruption should be attempted.

Due to their ookinete surface localization pattern, CPW-WPC family proteins may be potential candidates for transmission-blocking antigens, regardless of whether CPW-WPC-1 is dispensable in parasite transmission to mosquitoes. Previous reports have demonstrated that specific antibodies against P25 and P28 inhibited parasite transmission in mosquito midguts, although single disruption of each gene showed no clear defects during mosquito-stage development [12,30]. Moreover, CPW-WPC proteins are conserved among *Plasmodium* species, including *P. falciparum* and *Plasmodium vivax*, and CPW-WPC family proteins are less polymorphic than known blood-stage vaccine targets, such as apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1). These characteristics are clinically important for vaccine development; therefore, further studies including transmission-blocking assays using specific antibodies against CPW-WPC family proteins should be required.

**Conclusions**

The current study describes PyCPW-WPC-1, a novel post-transcriptionally regulated ookinete surface protein. This protein contains CPW-WPC domains, composed of conserved cysteine residues. *In silico* analysis shows that there are eight members of the CPW-WPC protein family, all of which are regulated by DOZI, a post-transcriptional regulatory factor that functions during parasite development in mosquitoes. By targeted gene disruption, it is demonstrated that PyCPW-WPC-1 is dispensable during ookinete development and invasion of midgut epithelium, which may suggest the redundancy of CPW-WPC-WPC family proteins. The identification of this novel ookinete surface protein family could be useful for understanding biology of mosquito-stage parasite development and for detecting possible candidates for transmission-blocking vaccine targets.
Additional files

**Additional file 1:** Gene-specific primers for transcriptional profiling.  
**Additional file 2:** Localization of PY03515 on the zygote/ookinete surface.

**Abbreviations**
- TBV: Transmission-blocking vaccine; DOZI: Development of zygote inhibited; CITH: Homolog of worm CAR-I and fly Trailer Hitch; CPM: Incomplete medium; OCM: Oocyste culture medium; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS: Phosphate-buffered saline; Pt: Protease inhibitors; cDNA: Complementary DNA; PCR: Polymerase chain reaction; GST: Glutathione S transferase; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HRP: Horseradish peroxidase; IFA: Indirect immunofluorescent assay; DAPI: 4′,6-diamidino-2-phenylindole.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
NK, TT, SR, MTR, and TI conceived, designed, and coordinated the study. NK, MTC, and RJ participated in the acquisition of data. NK, TT, SR, MTR, and TI analysed and interpreted the data. NK drafted the first version of the manuscript, and NK, MT, and TI were involved in critically revising the manuscript. All the authors have read and approved the final manuscript.

**Acknowledgements**
This study was supported by JSPS KAKENHI (grant number 21406010) to MT and JSPS KAKENHI (grant number 22406007) to TI.

**Author details**
1Department of Molecular Parasitology, Graduate School of Medicine, Ehime University, Shitsukawa, Toon, Matsuyama, Ehime 791-0295, Japan.  
2Division of Molecular Parasitology, Proteo-Science Center, Ehime University, Shitsukawa, Toon, Matsuyama, Ehime 791-0295, Japan.  
3Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Ehime 790-8577, Japan.  
4Department of Pathobiology, Faculty of Science, Mahidol University, Ratchatevi, Bangkok 10400, Thailand.  
5Present address: Department of Pathobiology, Faculty of Science, Mahidol University, Ratchatevi, Bangkok 10400, Thailand.  
6Present address: Mahidol Viva Research Unit, Faculty of Tropical Medicine, Mahidol University, Ratchatevi, Bangkok 10400, Thailand.

Received: 21 September 2012 Accepted: 8 April 2013 Published: 15 April 2013

**References**
1. World Health Organization: World Malaria Report. Geneva, Switzerland: World Health Organization; 2011.
2. Murray CJL, Rosenfeld LC, Lim SS, Andrews KG, Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez AD: Global malaria mortality between 1980 and 2010: a systemic analysis. Lancet 2012, 379:413–438.
3. Schwartz L, Brown GV, Genton B, Moorthy VS: Critical points of therapy for malaria. Curr Opin Infect Dis 2009, 22:560–569.
4. Breman JG, Brannand-Bennett AD: The challenge of malaria eradication in the twenty-first century: Research linked to operations is the key. Vaccine 2011, 29S597–D103.
5. Greenwood BM, Ridock DA, Hyle DE, Kappe SH, Alonso PL, Collins FH, Duffy PE: Malaria: progress, perils, and prospects for eradication. J Clin Invest 2008, 118:1566–1576.
6. Genton B: Malaria vaccines: a tool for travellers or a tool for eradication? Expert Rev Vaccines 2008, 7:599–611.
7. Carter R: Transmission blocking malaria vaccines. Vaccine 2001, 19:2369–2374.
8. Ghosh A, Edwards MJ, Jacobs-Lorena: The journey of the malaria parasite in the mosquito: Hope for the new century. Parasitol Today 2000, 16:196–201.
9. Carter R, Kumar N, Quakly I, Good M, Mensah K, Graves P, Miller L: Immunity to sexual stage of malaria parasites. Prog Allergy 1988, 41:193–214.
10. Tsuboi T, Greenwood BM: Malaria vaccines and their potential role in elimination of malaria. Malar J 2008, 7:Suppl 1:510.
11. Scholz SW, Simon N, Lavazec C, Dude MA, Templeton TJ, Pradel G: PICCP proteins of Plasmodium falciparum: gametocyte-specific expression and role in complement-mediated inhibition of exflagellation. Int J Parasitol 2008, 38:327–340.
12. Tsuboi T, Gao Y, Hitsumoto Y, Yanagi T, Kanbara H, Torii M: Two antigens on zygotes and ookinetes of Plasmodium yoelii and Plasmodium berghei that are distinct targets of transmission-blocking-immunity. Infect Immun 2006, 74:2260–2264.
13. Biller O, Dechamps S, Tewari R, Weng G, Franke-Fayard B, Bitlimann V: Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in malaria parasite. Cell 2004, 117:503–514.
14. van Dijk MR, Janse CJ, Thompson J, Waters AP, Braks JA, Dodemont HJ, Stunnenberg HG, van Gemert G, Sauerwein RW, Elng W: A central role for MB4/45 in malaria parasite male gamete fertility. Cell 2001, 104:153–164.
15. Liu Y, Tewari R, Ning J, Blagborough AM, Garborn S, Pei J, Grimih NW, Steele RE, Sinden RE, Snell WJ, Biller O: The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes Dev 2008, 22:1051–1068.
16. Hiral M, Aran M, Mori T, Miyagishima S, Kawai S, Kita K, Kuwata T, Terenius O, Matsutaka H: Male fertility of malaria parasites is determined by GCS1, a plant-type reproduction factor. Proc Natl Acad Sci USA 2007, 104:1655–1660.
17. Angriano F, Tan Y, Sturm A, Baum J: Malaria parasite colonisation of the mosquito midgut – Placeing the Plasmodium oococite centre stage. Int J Parasitol 2012, 42:519–527.
18. Mair GR, Braks JAM, Garver LS, Wiegant JACG, Hall N, Dirks RW, Khan SM, Dimopoulos G, Janse CJ, Waters AP: Regulation of sexual development of Plasmodium by transcriptional repression. Science 2006, 313:667–669.
19. Mair GR, Lasonder E, Garver LS, Franke-Fayard BMD, Carret CK, Wiegant JACG, Dirks RW, Dimopoulos G, Janse CJ, Waters AP: Universal features of post-transcriptional gene regulation are critical for Plasmodium zygote development. PLoS Pathog 2010, 6:1–12.
20. Plasmodium Genomics Resource: http://plasmodb.org/plasmo.
21. Tsuboi T, Takedo S, Inoko H, Jin L, Tsuchimochi M, Matsuda S, Han E, Otsuki H, Kaneko O, Sattabongkot J, Udomsangpetch R, Sasawaki T, Torii M, Endo Y: Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. Infect Immun 2008, 76:1702–1708.
22. Sasawaki T, Ogasaarava T, Morishita R, Endo Y: A cell-free protein synthesis system for high-throughput proteomics. Proc Natl Acad Sci USA 2002, 99:14652–14657.
23. Takai K, Sasawaki T, Endo Y: Practical cell-free protein synthesis system using purified wheat embryos. Nat Protoc 2010, 5:227–238.
24. Otsuki H, Kaneko O, Thongkukkutk A, Tachibana M, Inoko H, Takedo S, Tsuboi T, Torii M: Single amino acid substitution in Plasmodium yoelii erythrocyte ligand determines its localization and controls parasite virulence. Proc Natl Acad Sci USA 2009, 106:7167–7172.
25. Aurrecoechea C, Breteillot J, Brunk BP, Carlton JM, Dommer J, Fischer S, Sajja B, Gao X, Gingle A, Grant G, Harb OS, Heiges M, Innamorato F, Iodice J, Kissinger JC, Kraemer E, Li W, Miller JA, Morrison HG, Nayak V, Pennington C, Pinney DF, Roos DS, Ross C, Stoeckert CJ Jr, Sullivan S, Treatman C, Wang H: PlasmoDB: a functional genomic database for malaria parasites. Nucleic Acids Res 2009, 37:D539–D543.
26. Gissot M, Ting L, Daly TM, Bergman LW, Sinnis P, Kim K: High mobility group protein HMG2B is a critical regulator of Plasmodium oocyst development. J Biol Chem 2008, 283:17030–17038.
27. Bushell ESC, Ecker A, Schlegelmich T, Goulding D, Dougan G, Sinden RE, Christophides GK, Kafatos FC, Vlachou D: Paternal effect of the nuclear formine-like protein MSF1T on Plasmodium development in the mosquito vector. PLoS Pathog 2009, 5:1–12.
28. Ansari FA, Kumar N, Subramaniam MB, Gnanamani M, Ramachandran S: MAAP: Malaria adhesins and adhesion-like proteins predictor. Proteins 2008, 70:659–666.
29. Kim K: Malaria var gene expression: keeping up with the neighbors. Cell Host Microbe 2012, 11:1–2.
30. Tommas AM, Nargos G, Dimopoulos G, van Lin KH, Koning-Ward TF, Sinha R, Lupetti P, Beetsma AL, Rodriguez MC, Karras M, Hager A, Mendoza J, Butter GA, Kafatos F, Janse CJ, Waters AP, Sinden RE: P52 and P28 proteins of the malaria oocyst surface have multiple and partially redundant functions. EMBO J 2001, 20:3975–3983.

Cite this article as: Kangwanrangsan et al.: A member of the CPW-WPC protein family is expressed in and localized to the surface of developing ookinetes. Malaria Journal 2013 12:129.

doi:10.1186/1475-2875-12-129