as a terminal peroxidase that reduces hydrogen peroxide and organic hydroperoxides with the use of electrons donated by the thioredoxin (Trx) system [11,12]. With respect to the cellular functions of 2-Cys Prx, diverse findings have been reported in mammals, including modulation of cytokine-induced hydrogen peroxide levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation, and apoptosis [9,12,14].

Malaria parasites do not possess catalase or genuine GSH peroxidase in their genome, but the parasites are equipped with a 1-Cys Prx and two typical 2-Cys Prxs [1,2]. Thus, it is believed that GSH itself is the major redox buffer for transient H_2O_2 exposure and that the basal cellular peroxide flux is dealt with by the Trx system, which includes Prxs [7]. The 1-Cys Prx and one of the 2-Cys Prxs are expressed in the cytosol, and the other 2-Cys Prx is localized in mitochondria [15]. We recently reported that disruption of the gene encoding the cytosolic 2-Cys Prx (PlasmoDB; PF14_0368; PfTPx-1) in *P. falciparum* renders parasites hypersensitive to ROS and reactive nitrogen species, although it did not affect parasite growth under normal culture conditions [16]. This suggests that this Prx is not essential for parasite survival under culture conditions and that physiologic function of Prx in parasite cells remains to be elucidated. This Prx is constitutively expressed during the asexual development as well as in the gametocyte both in *P. falciparum* [17] and in the rodent malaria parasites [18,19]. To determine the cellular function of 2-Cys Prx in malaria parasites, we disrupted the gene encoding TPx-1 in the rodent malaria parasite *P. berghei* and analyzed the phenotype of the gene-disrupted parasite in infected mice. The disruptant showed normal asexual proliferation in mouse erythrocytes but showed a defect in gametocyte development, the sexual stage for transition to mosquitos that is induced by host environmental factors.

2. Material and methods

2.1. Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The parasite was maintained by mosquito transmission in *Anopheles stephensi* interspersed by a maximum of two serial passages in DBA/2 or Balb/c mice (Clea Japan, Tokyo, Japan). The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation of the International Medical Center of Japan.

2.2. Plasmid construction

For disruption of the PbTPx-1 gene, 5′ and 3′ portions of the gene were amplified by polymerase chain reaction (PCR) and cloned into the targeting vector pMD204 [20], which was supplied by the Malaria Research and Reference Reagent Resource Center; MR4/ATCC, Manassas, VA, USA. Each fragment contained part of the coding sequence and flanking region was amplified with sequence-specific primers and parasite genomic DNA. The primers used for the 5′ fragment were 5′-GG GGG CCC TCA CCA GCC TTA TTA AG-3′ and 5′-CC CCC GTC GACAAT ATA TTT CTT TCC-3′ (*Apa*I and *Hin*cII sites are underlined). The primers used for the 3′ fragment were 5′-CG GAA TTC CGA GTT TGT AAA AGA AC-3′ and 5′-TT CTG CAG TCA TTT AAA ATA AAG-3′ (*Eco*RI and *Pst*I sites are underlined). The primers were designed on the basis of sequences in the *P. berghei* genome database provided by the *Plasmodium* Genome Resource (PlasmoDB; PB_5804.1 and PB000037.01.0). PCR products were purified and cloned into upstream or downstream of the pyrimethamine-resistant form of *P. berghei* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, which was used as a selectable marker. For gene targeting experiments, the plasmid was digested with *Apa*I and *Pst*I to separate the linear targeting construct from the plasmid backbone.

2.3. Transfection and selection of disruptant

Transfection and selection were performed essentially according to the protocols of Ménard and Janse [21]. Blood-stage parasites used for transfection were prepared from Jcl:Wistar rats (Clea Japan) that had been infected by intraperitoneal (i.p.) injection of 5×10^6 parasitized erythrocytes per animal. Animals were killed when parasitemia reached 3%. Blood was drawn from the animal into heparinized syringes (100 μg/ml of blood) by cardiac puncture under ether anesthesia. Samples were washed for 10 min at $160 \times g$ at room temperature (RT) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM HEPES and 20% heat-inactivated fetal calf serum. The packed cells were then resuspended in 40–50 ml (10 volumes of the blood sample) of the medium and cultured in glass petri dishes (20–25 ml of the blood suspension/dish) in a AnaeroPack® microaerophilic culture system, which provided 5% CO_2 and 8% O_2 gases condition (Mitsubishi Gas Chemical, Tokyo, Japan), for 16 h at 37 °C with gentle rotation (50 rpm). Blood suspensions in the dishes were then layered onto 7.5 ml Nycoprep™ density gradient 1.077 medium (Axis-Shield, Oslo, Norway) and centrifuged first at $160 \times g$ for 5 min and then at $360 \times g$ (by shifting to high speed before stop) for 15 min at RT. Schizonts at the interface were collected and washed with 40 ml culture medium for 5 min at $360 \times g$ at RT and then with 3 ml phosphate-buffered saline (PBS). Schizont pellets were resuspended in 400 μl PBS containing 50 μg linearized targeting vector, then transferred to 0.4 cm electroporation cuvettes for transfection. Mixtures were subjected to an electric pulse (800 V, 25 μF) with a Gene Pulser® II System (Bio-Rad, Hercules, CA, USA) and were immediately inoculated into two rats (200 μl electroporated schizont suspension/animal) by intravenous (i.v.) injection. Animals were treated with pyrimethamine (12.5 mg/kg body weight) at 30 h after inoculation every 12 h until parasites were no longer detected in the blood circulation. When the parasitemia increased again to 1–2%, rats were treated with the same dose of pyrimethamine, and resistant parasites were transferred to new rats in which the parasites multiplied in the absence of drug. When parasitemia had reached 1%, the parasite population in

each rat was separated into wild-type and disruptant by limiting dilution. Both parasite genomes were determined by PCR and Southern blot analysis.

2.4. Nucleic acid techniques

Parasite-infected blood was passed through a CF11 (Whatman, Maidstone, Kent, UK) column and span with Lymphoprep^{TM} density gradient medium (Axis-Shield, Oslo, Norway) to remove leukocytes. Parasite-infected erythrocytes were then washed with PBS and lysed with PBS containing 0.05% saponin. Parasite pellets were washed several times with PBS, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Nucleic acids were extracted from parasite pellets. For Southern blot, genomic DNA was extracted by the standard phenol–chloroform method and incubated with *Spe*I. For Northern blot, total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Nucleic acids were separated by agarose gel electrophoresis and transferred onto HyBond N$^+$ membranes (Amersham Biosciences UK Ltd., Little Chalfont, UK). Membranes were probed with a 368-bp fragment spanning the 5′ sequence of *pbtpx-1* for Southern blot (Fig. 1B) and the entire coding sequence for Northern blot (Fig. 2B). The probes were labeled, hybridized, and detected with the AlkPhos Direct Labeling and Detection System (Amersham Biosciences).

2.5. Western blot analysis and indirect immunofluorescence assay

For Western blot analysis, parasite pellets were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 5% 2-mercaptoethanol [22]. After separation by SDS-PAGE (12.5%), parasite proteins were transferred electrophoretically to polyvinylidene difluoride sheets (Immobilon; Millipore, Bedford, MA, USA) and incubated with anti-recombinant PfTPx-1 (rPfTPx-1) rabbit IgG (25 μg/ml). Interspecific crossreactivity between PbTPx-1 and anti-rPfTPx-1 rabbit IgG has been reported [18]. Immune complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH, USA). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Indirect immunofluorescence assay was performed as described previously [15]. The IgG fraction of rabbit antisera to rPfTPx-1 was used at 20 μg/ml. Parasite cells on slides were mounted with Prolong AntiFade Solution (Molecular Probes, Eugene, OR, USA) and observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

2.6. Analysis of parasite development in mice

Five-week-old female Balb/c and C57B6 mice (Clea Japan) were infected with *P. berghei* (10^6 parasitized cells per mouse) by i.p. injection. Parasitemia and gametocytemia were determined by microscopic examination of Giemsa-stained thin blood films. Male and female gametocytes were distinguished by size and coloration. Numbers of exflagellation centers were counted according to a previously described method [23]. Accumulation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the parasite genome was evaluated with a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan) [24]. DNA for ELISA was extracted from parasites collected from infected mice (blood from 10 infected mice was pooled) when

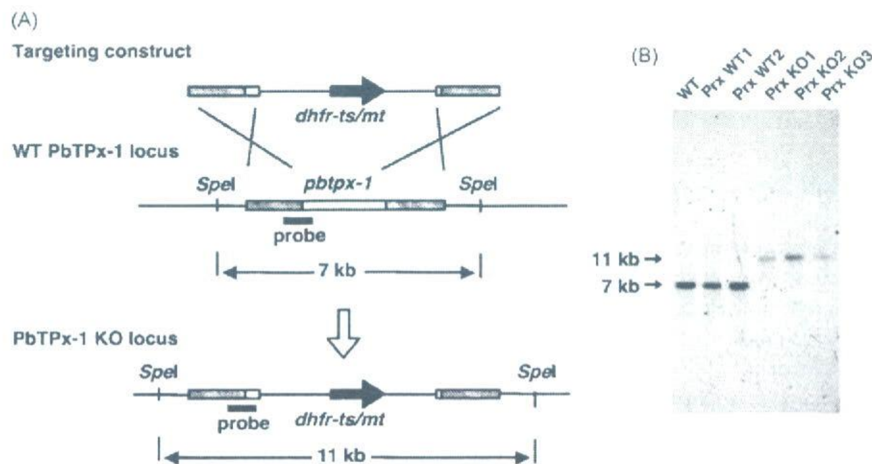

Fig. 1. Targeted disruption of the TPx-1 gene in *P. berghei*. (A) The targeting construct, composed of the 5′ and 3′ regions (hatched boxes) and partial open reading frame (open box) of the PbTPx-1 gene (*pbtpx-1*) and the pyrimethamine-resistant variant of DHFR-TS gene of *P. berghei* (*dhfr-ts/mt*) as a selectable marker (filled arrow), was integrated into the wild-type (WT) PbTPx-1 locus by double-crossover homologous recombination. Recombination disrupts *pbtpx-1* and creates the locus containing *dhfr-ts/mt* (PbTPx-1 KO locus), which confers pyrimethamine resistance to disruptants. The *Spe*I restriction sites and the position of the probe used for Southern blot analysis in B are indicated. (B) Southern blot analysis of genomic DNA samples from the parent parasite (WT), two wild-type parasite populations with pyrimethamine resistance (*dhfr-ts/mt* at the DHFR-TS locus) (Prx WT), and three *pbtpx-1* disruptant populations (Prx KO). DNA samples were digested with *Spe*I, separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized with probe. The single 11-kb band in the Prx KO populations indicates *pbtpx-1* disruption, whereas the single 7-kb band in the WT and Prx WT populations indicates an intact PbTPx-1 locus.

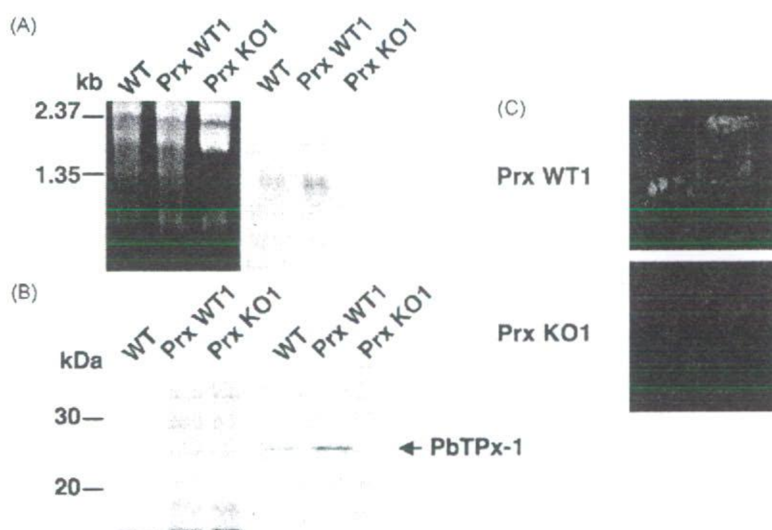

Fig. 2. Verification of the PbTPx-1 null phenotype in gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), Prx WT1 (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*), and Prx KO1 (*pbtpx-1* disruptant) were inoculated into mice, and parasite-infected erythrocytes, total protein, and total RNA of the parasite cells were prepared. (A) The absence of TPx-1-specific mRNA expression in Prx KO was examined by Northern blot analysis. Total RNA samples (20 μg) were separated on 1.5% agarose-formaldehyde gel (left panel), transferred to nylon membranes, and hybridized with probe (right panel). Ethidium bromide-stained gel showed equal loading. Molecular size markers in kb are indicated on the left. (B) The absence of TPx-1 protein in Prx KO was examined by Western blot analysis. Total protein samples (20 μg) were separated by SDS-PAGE (left panel) and probed with anti-TPx-1 antibody (right panel). Coomassie brilliant blue-stained gel showed equal loading. Protein size markers in kDa are indicated on the left. (C) The absence of TPx-1 protein in Prx KO was confirmed by indirect immunofluorescence assay. TPx-1 protein is stained in green, and parasite nuclei are stained in blue.

parasitemia reached approximately 4% (4–5 days after infection). Levels of 8-OHdG (ng/mg DNA) are expressed as the mean of triplicate measurements.

2.7. Statistical analysis

Differences were evaluated with Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Targeted disruption of the PbTPx-1 gene

Merozoites in segmented schizonts were transfected with the targeting construct by electroporation and were subsequently inoculated into naïve rats. Integration of the construct into the PbTPx-1 locus by homologous recombination resulted in disruption of the single-copy gene and insertion of a selectable marker, the DHFR-TS gene with a pyrimethamine-resistance mutation (*dhfr-ts/mt*), at the locus (Fig. 1A). Parasites with *pbtpx-1* disruption were selected by treatment with pyrimethamine. PCR and Southern blot analyses showed that parasites selected with pyrimethamine were a mixture of wild-type parasites and *pbtpx-1* disruptants (data not shown). Parasite populations were separated into two groups by limiting dilution and subsequent inoculation into 18–22 rats. In two independent electroporation experiments, six disruptant populations (Prx KO) were obtained and cloned. In the first experiment, three Prx KO populations (Prx KO1–3) and two wild-type parasite populations (Prx WT1 and 2) were obtained (Fig. 1B). In the second experiment, two Prx KO populations (Prx KO4 and 5) were obtained (data not shown). The *dhfr-ts* locus of Prx WT parasites was amplified by PCR and sequenced, and replacement with the pyrimethamine-resistance mutation was confirmed (data not shown). Northern blotting, Western blotting, and immunofluorescence assay confirmed the TPx-1-null phenotype of Prx KO1 (Fig. 2). This phenotype was also confirmed for all Prx KO populations (data not shown).

3.2. PbTPx-1 gene disruption does not affect asexual development

Prx KO1–3 and Prx WT1 and 2 were used to infect Balb/c mice, and the course of parasite development within erythrocytes was compared to that of the parent strain (WT) (Fig. 3A). Two Prx WT populations showed equal development and multiplication within erythrocytes, similar to WT, and they showed a high level of parasitemia (>5%) 5 days after infection. The courses of parasitemia observed in Prx KO1-infected mice were also similar to that in WT-infected mice. This phenotype was confirmed in Prx KO2 and 3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3B). There was no difference in the morphology of parasite cells between five Prx KO, two Prx WT, and WT populations (data not shown). These eight parasite populations did not kill mice until day 8 of observation. Prx KO1, Prx WT2, and WT populations each killed one of three mice, respectively, on day 9 of observation. It is known that Balb/c mice in some laboratories are resistant to the development of cerebral malaria (CM) induced by the *P. berghei* ANKA strain and do not develop lethal infection [25]. To determine the phenotype of *pbtpx-1* disruption in strains of mice with differing susceptibility to the *P. berghei*

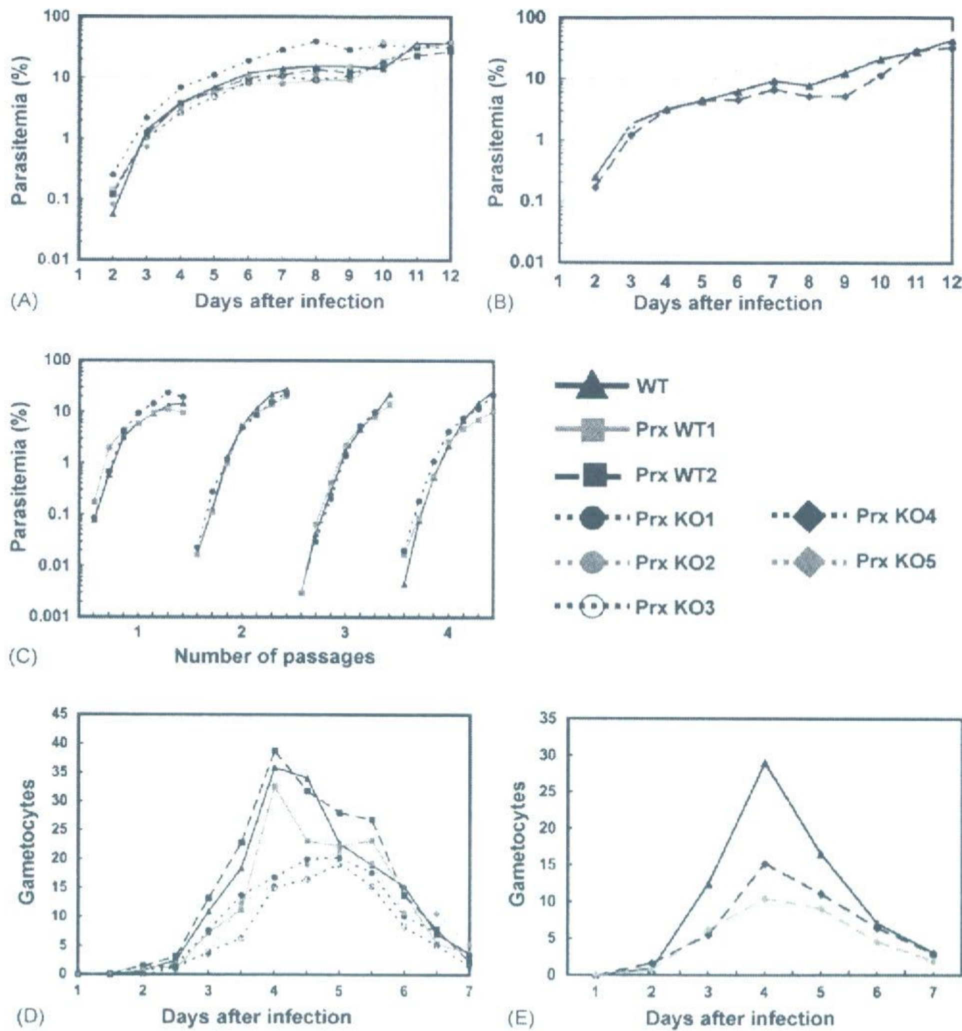

Fig. 3. Infection of mice with TPx-1 gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), two Prx WT (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*) and five Prx KO (*pbtpx-1* disruptant), were inoculated into mice, and the courses of parasite development in erythrocytes were observed. (A and B) Changes in parasitemia 12 days after infection (three mice per group: six groups in (A) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (B) WT and Prx KO4 and 5). (C) Changes in the course of parasitemia during four blood passages (two mice per group: three groups, WT, Prx WT1, and Prx KO1). (D and E) Changes in gametocytemia 7 days after infection (three mice per group: six groups in (D) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (E) WT and Prx KO4 and 5). Data are mean values of parasitemia percentage (A–C) and gametocyte number (gametocytes/10^4 erythrocytes) (D and E) in each group.

ANKA strain, C57B6 mice, which are known to develop CM and lethal infection [25], were infected with Prx KO1, and the course of parasite development within erythrocytes was compared to that of WT. Prx KO1 and WT multiplied equally and killed mice 7–8 days after infection. The morphology of parasite cells and the numbers of animals killed during 8 days of observation between the two parasite populations did not differ (data not shown).

A function of 2-Cys Prx in the prevention of damage to DNA by oxidative stress has been suggested in mammals [26]. Therefore, the cumulative effect of oxidative stress on parasite growth in vivo in the *pbtpx-1* disruptant was evaluated. For this purpose, Prx KO1, Prx WT1, and WT were continuously maintained in Balb/c mice by inoculating parasite-infected blood and comparing the course of parasitemia between passages. Blood passage was performed on the 7 day of infection and was repeated four times. The courses of parasitemia observed in Prx KO1-, Prx WT1-, and WT-infected mice from the first to the fourth passage are shown in Fig. 3C. The course of parasitemia after four passages compared to that after the first inoculation did not differ between the three parasite populations. Passage was repeated six more times, and the course of parasitemia observed for the three parasite populations at the 10th passage was similar to that observed at the fourth passage (data not shown). To determine whether *pbtpx-1* disruption enhances DNA damage in the parasite genome, the level of 8-OHdG, a marker of oxidative DNA damage, was compared between Prx KO1 and WT. The level of 8-OHdG in the parasite genome was similar between Prx KO1 (11.7 ng/mg DNA) and WT (9.1 ng/mg DNA), suggesting that *pbtpx-1* disruption does not affect the physiologic level of DNA oxidation. This result was confirmed in two independent experiments (data not shown).

3.3. PbTPx-1 gene disruption affects gametocytogenesis

To evaluate the effect of *pbtpx-1* disruption on development of sexual-stage parasites, the numbers of gametocytes in parasite-infected blood were counted and compared among Prx KO1–3, Prx WT1 and 2, and WT (Fig. 3D). In Prx WT1- and WT-infected mice, gametocytes were observed from 2 days after infection; they increased in number with parasitemia progression and peaked 4 days after infection. Gametocytemia, recorded as the number of gametocytes/10^4 erythrocytes in Prx WT1- and WT-infected mice at the day 4 of infection, was 32.5 ± 5.5 and 35.8 ± 11.6, respectively ($P = 0.34$). The course of gametocytemia between Prx WT1- and WT-infected mice did not differ. This phenotype was confirmed in two populations of Prx WT. In Prx KO1-infected mice, gametocytes were also observed from 2 days after infection and peaked 5 days after infection. The peak of gametocytemia in Prx KO1-infected mice was thus delayed 1 day from that of Prx WT1- and WT-infected mice. Gametocytemia, recorded in Prx KO1-infected mice as the number of gametocytes/10^4 erythrocytes on day 5 of infection, was 20.2 ± 0.9. The highest number of gametocytes was thus significantly lower ($P < 0.01$) in Prx KO1-infected mice than in WT-infected mice. The *pbtpx-1* disruption phenotype, in addition to the delay in gametocytemia peak, showed a significant decrease in the numbers of gametocytes ($P < 0.02$) in Prx KO1–3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3E).

To determine whether *pbtpx-1* disruption affects the male/female ratio of gametocytes or exflagellation activity of male gametocytes, Prx KO1, Prx KO4, Prx WT1, and WT were used to infect mice (Table 1). The male/female ratios of gametocytes in the three parasite populations at the day of peak gametocytemia were similar: 0.44, 0.53, and 0.64 for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 0.62 and 0.50 for Prx KO4- and WT-infected mice in experiment 2. This phenotype was confirmed in five populations of Prx KO (data not shown). The percentages of exflagellation, calculated according to the numbers of exflagellation centers and the numbers of male gametocytes in the blood at the day of peak gametocytemia, were 70, 64, and 76% for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 59 and 60% for Prx KO4- and WT-infected mice in experiment 2. Thus, the majority of male gametocytes observed in blood were equally viable in terms of exflagellation activity among the three parasite populations. This finding suggests that low gametocytemia in Prx KO is attributed to impaired development of gametocytes rather than to their decreased survival.

4. Discussion

The results presented here suggest that *pbtpx-1* disruption does not affect asexual intraerythrocytic growth of parasites. The results from passage experiments and evaluation of 8-OHdG suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx [26], and that it is not essential for asexual parasite growth in mouse erythrocytes. However, the fact that the parasite lacks catalase and genuine GSH peroxidase and that the major cytosolic peroxide-detoxifying capacity appears to be provided by Prx may contradict the present findings [1,2]. Microarray analysis of the disruptant and the parent parasite may provide insights with respect to redundancy in parasite antioxidant defenses and compensation for the lack of Prx. Our results may not indicate that the gene is inessential for asexual growth in *P. falciparum* because there are differences in the life cycle of the mammalian stage between *P. falciparum* and rodent malaria parasites. *P. falciparum* develops in erythrocytes sequestered in the microvasculature, where the parasite may experience more severe oxidative stress than do parasites in the blood circulation [5]. The parasite may require Prx for development under such stressful conditions. Such a possibility should be investigated in a monkey model of *P. falciparum* infection [27]. The relatively high level of 8-OHdG formation in the parasite genome compared to that in mammalian cells [24] may reflect the absence of nuclear Prx in this organism [1,2,9]. How malaria parasites deal with the accumulation of oxidative DNA damage is of interest; a hypothetical protein with putative excision DNA repair function or DNA glycosylase function (PF10835c) has been identified in the genome of the malaria parasite.

Table 1
Male/female ratios of gametocytes and exflagellation activity of male gametocytes in TPx-1gene-disrupted *P. beghei*[a]

	Experiment 1			Experiment 2	
	WT	Prx WT1	Prx KO1	WT	Prx KO4
Peak of gametocytemia					
Gametocyte number[b]	30 ± 5.5	37 ± 5.5	18 ± 2.5	31 ± 3.2	14 ± 1.6
Days after inoculation	4.0	4.0	4.5	4.0	4.5
Male/female ratio					
Day of peak gametocytemia	0.64	0.53	0.44	0.50	0.62
Day 5.5	1.06	1.07	0.92	1.01	1.15
Exflagellation (%)[c]					
Day of peak gametocytemia	76 (52–88)	64 (51–81)	70 (53–81)	60 (54–64)	59 (55–63)
Day 5.5	16 (10–20)	13 (7–27)	24 (9–54)	14 (12–17)	4.1 (0.6–10)

[a] Values are means ± standard deviations (gametocyte number), mean (male/femal ratio), and mean with range (exflagellation, %) calculated from data obtained from four mice.

[b] Gametocytes per 10^4 erythrocytes.

[c] Exflagellation (%) = (number of exflagellation centers in 10^4 erythrocytes/number of male gametocytes in 10^4 erythrocytes) × 100.

Nonetheless, the results presented here suggest that PbTPx-1 is required for normal gametocyte development; however, it does not affect the male/female ratio of gametocytes. We could not observe the defect in gametocyte development in *P. falciparum*, since we disrupted the gene in the FCR-3 strain, which produces only a little numbers of gametocyte [16]. Gametocytes are sexual-stage parasites involved in the transition between the mammalian host and the mosquito. This stage arises during asexual cycling, and it has generally been accepted that the trophozoites of the preceding asexual generation are already committed to either develop into gametocytes or continue asexual cycling [28]. Although the mechanism by which PbTPx-1 contributes to gametocyte development is unknown, ROS and antioxidants, including Prx, are known to influence the expression of a number of genes and to influence signal transduction pathways during cell differentiation and during organismal development [29,30]. A model in which Prx regulates peroxide-mediated signaling cascades by acting as a floodgate for H_2O_2 in mammalian cells has been proposed [12,14]. Homology modeling of PbTPx-1 based on structural alignment with human homologues showed that it possesses a redox-sensitive type of peroxidatic active site structure, which enables the enzyme to act according to the floodgate model (data not shown). A model in which 2-Cys Prx regulates activation of stress-activated mitogen-activated protein kinase (MAPK) (Sty1) by forming peroxide-induced disulphide complexes in yeast has also been proposed [31]. A molecule homologous to Sty1 (MAPK1) has been identified in the *P. falciparum* genome, although its function is unknown. Gametocyte development can be induced by environmental factors such as host factors or drug treatments, and there is consistent evidence for the involvement of signal transduction pathways in this process [28]. PbTPx-1 may be involved in transducing extracellular signals for gene expression and in initiation of gametocyte development. If this is the case, gametocyte development and its sexual determination may occur independently, with PbTPx-1 participating only in the former process. Alternatively, PbTPx-1 may contribute to protein synthesis in both male and female gametocyte development as a molecular chaperone. Evidence that Prx acts as a molecular chaperone in yeast and in human cells has recently been reported [32,33]. PbTPx-1 may not take part in the process of male gamete formation, including gender-specific signaling, because the disruptant retained normal exflagellation activity [34,35]. Whether it participates in female gametogenesis will require fertilization experiments in vitro and in the mosquitos [35,36]. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, the present study suggests the involvement of this moleclue in the sexual development of the malaria parasite. Further studies to elucidate the role of TPx-1 in gametocyte development will provide further insight into the involvement of this antioxidant protein in the sexual development of malaria parasites and may provide novel transmission-blocking strategies.

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LIMITED ALLELIC DIVERSITY OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN 1 GENE FROM POPULATIONS IN THE SOLOMON ISLANDS

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Abstract. Meiotic recombination generates allelic diversity in the *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) gene. In this study, we monitored recombination-based diversity of *msp1* in Guadalcanal, the Solomon Islands, where malaria transmission is high. We identified 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types), and compared them with those from areas of low transmission in Thailand and Vanuatu. The mean number of 5′ recombinant types per person (multiplicity) was lower in Guadalcanal than in Thailand. Guadalcanal populations had 6–8 *msp1* haplotypes; the numbers are comparable to Vanuatu but much lower than in Thailand. There were marked geographic differences in distribution of *msp1* haplotypes. Linkage disequilibrium in *msp1* was stronger in Guadalcanal than in Thailand, suggesting limited recombination events in the Solomon Islands. We suggest that the frequency of recombination events in *msp1* is determined not only by transmission intensity but by the number of *msp1* alleles prevalent and multiplicity of infections.

INTRODUCTION

One of the obstacles to development of effective vaccines against the human malaria parasite *Plasmodium falciparum* is the diversity of vaccine candidate antigens in natural parasite populations. Evidence indicates that antigen diversity limits the efficacy of acquired immunity based on strain-specific anti-parasite immunity.[1–3] The diversity of antigen genes differs greatly among different areas endemic for malaria.[4–6] Therefore, it is important to investigate geographic differences in the genetic diversity of *P. falciparum*. The merozoite surface protein 1 (MSP-1) of *P. falciparum* is a major blood-stage surface antigen.[7] The gene (*msp1*) consists of 17 blocks according to the degree of sequence conservation among alleles.[8,9] Sequence variation in *msp1* is mainly dimorphic (two major allelic forms) in all variable blocks other than block 2, which is trimorphic (three allelic forms) (Figure 1). The block is grouped according to the presence or absence of nine-basepair repeats and the type of repeating sequences. MSP-1 contains at least two regions of protective immune target: block 2 near the N-terminus and block 17 at the C-terminus. In an area of Africa highly endemic for malaria, human antibodies against block 2 are associated with clinical immunity in an allelic-type-specific manner.[10] Block 17 encodes a C-terminal 19-kD polypeptide fragment that is produced by processing of MSP-1 at the merozoite surface. Antibodies against this 19-kD fragment inhibit merozoite invasion into red blood cells,[11,12] and immunization with the 19-kD fragment confers protection against challenging infections in animals.[13] Invasion-inhibiting antibodies against the 19-kD fragment have been detected in sera from individuals living in areas highly endemic for malaria.[14,15]

Meiotic recombination is a major genetic mechanism in the generation of allelic diversity in *msp1*.[8,16] The frequency of recombination events in *P. falciparum* depends primarily on the frequency of its transmission because *P. falciparum* undergoes meiotic recombination at the mosquito stage. We have been using recombination-based haplotypes of *msp1* as a polymorphic genotype marker to study allelic diversity of *msp1* in parasite populations.[17–19] The *msp1* haplotypes are defined as unique associations of 5′ recombinant types (blocks 2–6) and 3′ sequence types (block 17). In analysis of linkage disequilibirum, the prevalence of *msp1* haplotypes acts as an indicator of the frequency of recombination events in parasite populations. In a high-transmission area, there is little linkage disequilibrium in the *msp1* sequence,[20] whereas there are moderate levels of linkage disequilibrium in mesoendemic areas such as Thailand and Vietnam.[21] In contrast, there are very high levels of linkage disequilibrium in Brazil, a hypoendemic area.[22] However, nearly complete linkage disequilibrium occurs in Vanuatu in the southwestern Pacific,[19] where the level of malaria transmission is comparable to that of Southeast Asia, suggesting that the frequency of transmission is not the sole factor determining frequency of recombination events in *msp1*.

In the present study, we examined *P. falciparum* populations from Guadalcanal, the Solomon Islands, which are located between Papua New Guinea and Vanuatu. Malaria is highly endemic in the Solomon Islands, and malaria transmission is more intense than in Vanuatu. *Plasmodium falciparum* populations in the Solomon Islands are the most likely source of the Vanuatu populations because Vanuatu is located at the southern margin of the malarious band extending from Papua New Guinea through the Solomon Islands, and the Solomon Islands are the only neighboring country where malaria is prevalent. Therefore, it would be highly instructive to investigate the diversity of *msp1* haplotypes and linkage disequilibrium in *msp1* in populations from the Solomon Islands, and compare them with those of populations from Vanuatu and other geographic areas. The present results show that the diversity of *msp1* haplotypes is greater in Guadalcanal than in Vanuatu, as expected. In contrast, despite high endemicity, the diversity in the Solomon Islands was relatively limited, compared with populations from Thailand, and linkage disequilibrium in *msp1* was greater in the Solomon Islands than

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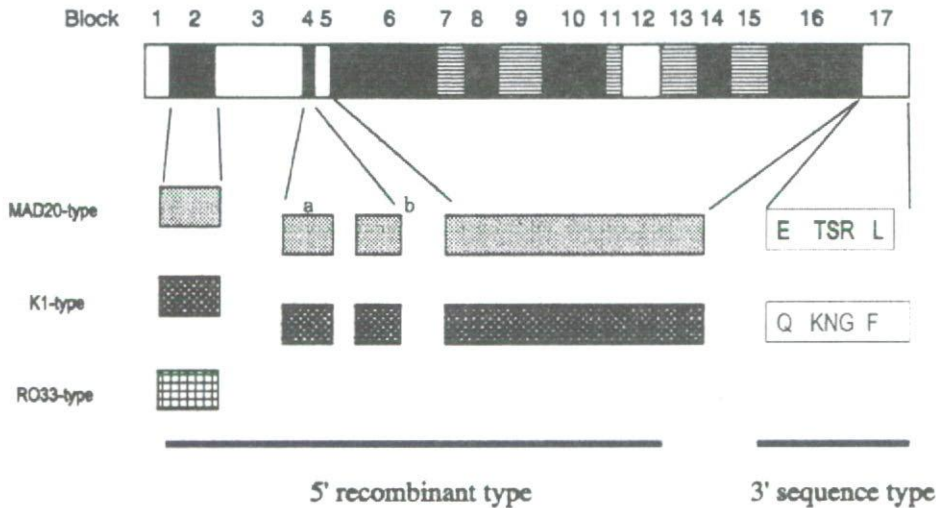

FIGURE 1. Structure of the *Plasmodium falciparum* merozoite surface protein 1 gene and the strategy for determination of 5′ recombinant type and 3′ sequence type. Inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For variable blocks, MAD20-type, K1-type, and RO33-type sequences are represented by half-tone, checkerboard, and cross-striped bars, respectively. The 5′ recombinant type was determined by polymerase chain reaction (PCR) amplification of blocks 2–6 using allelic type-specific primers of blocks 2 and 6, followed by a nested PCR for blocks 4a and 4b using allelic specific primers of blocks 4a and 4b. The five amino acid substitutions in block 17, indicated by the one-letter codes, were determined by sequencing. The 3′ sequence type is the combination of those residues.

in Thailand. Also, all of our Solomon Islands isolates had the Papua New Guinea-type *P. falciparum* chloroquine resistance transporter (pfcrt),[23] the locus that determines chloroquine resistance in *P. falciparum.*

MATERIALS AND METHODS

Study area and sample collection. *Plasmodium falciparum* field isolates were collected from northern Guadalcanal, the Solomon Islands, in the southwestern Pacific, from July 1995 to March 1996. Guadalcanal is the largest island in the Solomon Islands with a population of 409,000 (1999 census), and has an area of 5,336 km^2, a length of 160 km, and a width of 48 km (Figure 2). Malaria in Guadalcanal is hyperendemic, with parasite-positive rates of 45% and 39% in 1994 and 1995, respectively.[24] Transmission of malaria is perennial, with a peak during the rainy season from December to May. Both *P. falciparum* and *P. vivax* are prevalent, and approximately two-thirds of the parasites are *P. falciparum*.

Ninety isolates of *P. falciparum* were collected from three areas in northern Guadalcanal (Figure 2): 40 clinical isolates from outpatients of a hospital in Honiara City (area A), the capital of the Solomon Islands; 26 isolates from 3 villages (Kaotave, Tadhimboko, and Nugalitav) in area B; and 24 isolates from 1 village (Ruavatu) in area C. Area C is directly connected to area A by a road, whereas the three villages in area B are located several kilometers from the road. In rural areas B and C, samples were collected from participants of malariometric surveys, and most of the donors were primary school children 8–15 years of age with a mean age of 13.2 years in area B (range = 6–59) and a mean age of 14.9 years in area C (range = 11–18). Parasite-positive individuals were asymptomatic in most cases in areas B and C. Approximately half of the clinical samples from Honiara were collected from subjects more than 15 years of age, with a mean age of 18.3 years (range = 4–60). Giemsa-stained thick blood smears were examined microscopically, and *P. falciparum*-positive blood was withdrawn using a syringe containing EDTA and stored at −60°C. Parasite genomic DNA was extracted from blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[25] Informed consent was obtained from the donors and the school principals. This study was reviewed and approved by the Ethical Committee of the Solomon Islands for Medical Research.

Samples collected in January 1994 (n = 92)[26] were also included in this study. These samples were from area B and included three villages (Talaura, Tadhimboko, and Kolona). The mean age of the donors was 21.6 years (range = 5–89). In addition, we used clinical isolates (n = 111) from patients who attended a malaria clinic in Mae Sot in northwestern Thailand in 1995.[18] The mean age of the donors in Thailand was 24.6 years (range = 13–48). Isolates from Vanuatu (n = 164) were collected during malariometric surveys in rural villages on four islands[19]: Namasari and Ontar in eastern and western Gaua island, respectively, in 1997, Big Bay in northern Santo island in 1997, Baie Barier and Pangi in eastern and western Pentecost island, respectively, in 1998, and Lingarak and Brenwe/Leviamp in eastern and northwestern Malakula island, respectively, in 1996 and 1998. Subjects were primarily school children in each village with a mean age of 6.9 years (range = 0–25), 12.6 years (range = 2–47), 7.9 years (range = 2–30), 10.6 years (range = 3–36), and 9.8 years (range = 1–28) in Gauaisland, Santo island, Pentecost island, Malakula island in 1996, and Malakula island, respectively, in 1998. The overall parasite-positive rate was 14%. *Plasmodium falciparum* infections detected during surveys were asymptomatic in most cases.

Determination of *msp1* haplotypes. The *msp1* haplotypes are defined as unique associations of 5′ recombinant types

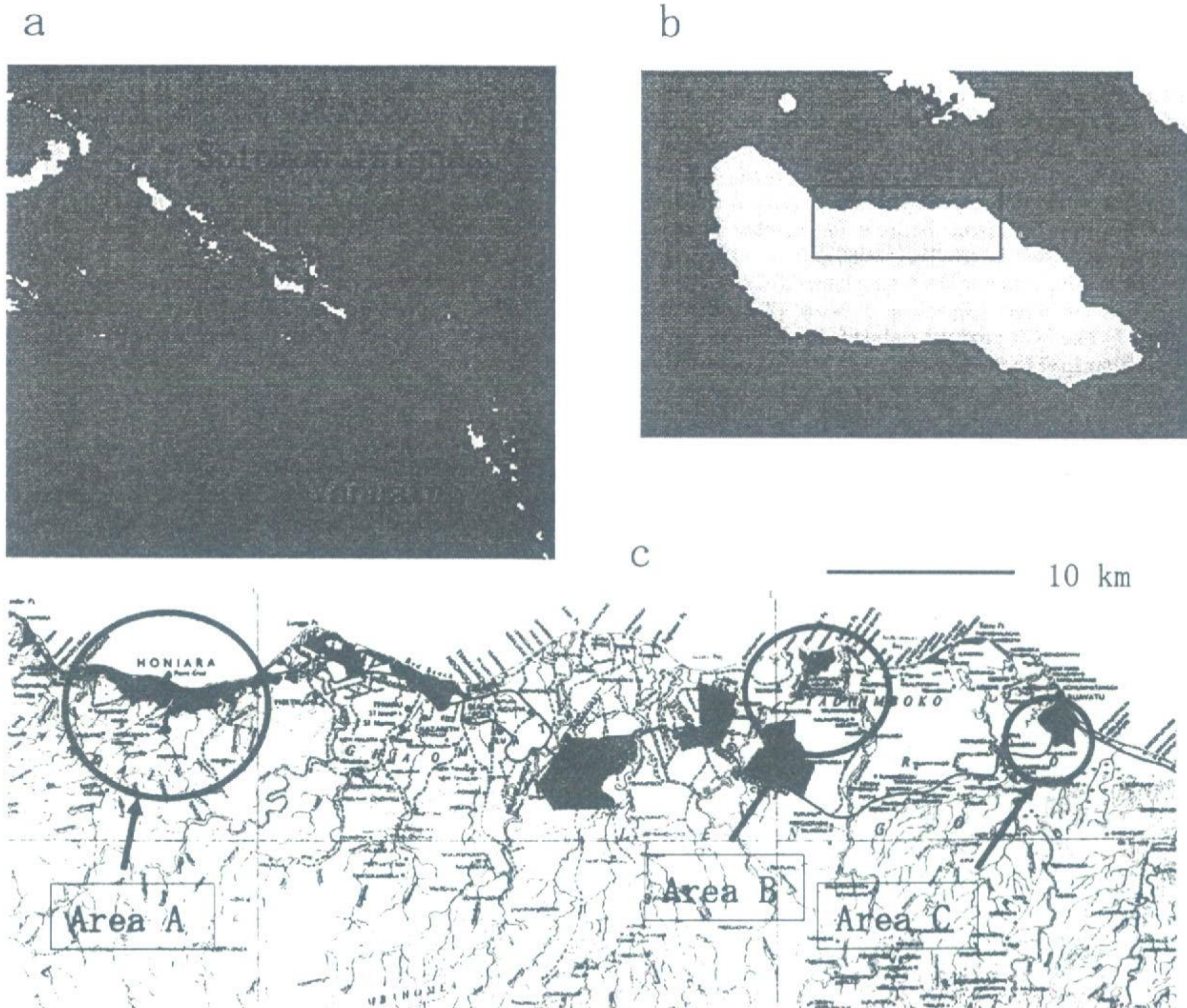

FIGURE 2. Location of study area. **a**, Solomon Islands and Vanuatu. The box shows Guadalcanal. **b**, Guadalcanal. The box shows the study area in northern Guadalcanal. **c**, The three study areas A, B and C are shown by the circles.

and 3′ sequence types (Figure 1). The methods used to determine 5′ recombinant types and 3′ sequence types were as follows.

The 5′ recombinant types (block 2–6) of *P. falciparum msp1* (a 5-kb gene) consist of 17 distinct sequence blocks (Figure 1). Sequences of all variable blocks other than block 2 are dimorphic, either one or the other of two major allelic types: K1 and MAD20. Block 2 is represented by one of three major types: K1, MAD20, and RO33. The 5′ recombinant types comprise unique combinations of allelic types of blocks 2, 4a, 4b, and 6. To date, no recombination has been found in blocks 6–16.[18,27] Determination of 5′ recombinant types involves primary amplification of a 1-kb region spanning blocks 2–6, followed by nested PCR amplification of block 4a–4b (approximately 100 basepairs). Procedures for determination of 5′ recombinant types are described elsewhere.[26] For the PCR, we used 1 μL of template DNA that was adjusted to be equivalent to 1 μL of the original blood.

We previously reported *msp1* 5′ recombinant types from a Thai population.[18] In that study, 1 μL of 20-fold diluted DNA was used for the PCR, which is an amount not equivalent to the original volume of blood. Furthermore, we later found that the typing method used in that study was neither very sensitive nor specific, and that it produced artifacts due to the relatively weak specificity of the PCR primers used. We therefore improved that method to obtain higher sensitivity and specificity and to avoid artifacts.[26] In the present study, we used the improved method to re-type 5′ recombinant types of the Thai samples. Although there was no significant difference in distribution of 5′ recombinant types between the two methods, the improved method substantially increased the rate of multiple infections of distinct 5′ recombinant types (65% versus 30%) and the mean number of 5′ recombinant types per isolate (3.6 versus 1.8). Determination of 5′ recombinant types was successful in 82 of 90 isolates from the Solomon Islands samples collected in 1995 and 1996 and in 107 of 111 isolates from the Thai samples. These were compared with the 5′ recombinant types previously determined for Solomon Islands isolates collected in 1994 (n = 84)[26] and Vanuatu isolates in 1996–1998 (n = 141).[19]

The 3′ sequence type (block 17), nucleotide sequence of the 3′ region of *P. falciparum msp1*, which encodes the C-terminal 19-kD polypeptide, was determined by direct sequencing. To obtain a clear sequence, we selected only isolates with mono-infections of a single 5′ recombinant type, and excluded isolates of multiple infections. The number of samples sequenced was 30 for the samples collected in 1994 from Guadalcanal, 47 for the samples collected in 1995 and 1996 from Guadalcanal, and 48 for the samples from Thailand. Because the number of mono-infections was limited in our Thai samples (n = 4), it was increased by diluting genomic DNA templates (20-fold). The procedures used for direct sequencing of block 17 are described elsewhere.[19] The PCR products coding for amino acid residues from position 1637 to the C-terminus of MSP-1 (positions are according to Miller and others[9]) were sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Sequences were verified by re-sequencing PCR products independently amplified from the same DNA. To date, five amino acid changes have been identified in block 17 in samples obtained from various geographic areas (E or Q at amino acid residue 1644, T or N at 1691, SR or NG at 1700–1701, and L or F at 1716)[21,28] (Figure 1). Hereafter, we refer to combinations of these residues as the 3′ sequence type.

Sequencing of *pfcrt*. Polymorphism at amino acid residues 72–76 in the second exon of *pfcrt*[29] was determined for the Solomon Islands isolates with mono-infection of a 5′ recombinant type from samples collected in 1994 (n = 30) and samples collected in 1995 and 1996 (n = 47). A DNA fragment (468 basepairs) covering the second exon of *pfcrt* was amplified using the method used above for *msp1* with primers CQF4 (5′-CAGATGGCTCACGTTTAGGTGGAGGT-3′) and CQR4 (5′-TGTGTAATGTTTTATATTGGTAGGTGGA-3′), in a 50-μL reaction mixture containing 1 μL of template genomic DNA. The PCR product was purified and directly sequenced using CQF4 as a sequencing primer.

Statistical analyses. Frequency distributions of *msp1* 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes in different parasite populations were compared using the chi-square test with Yates' correction for data sets less than 10 and Fisher's exact test. Differences in the rate of multiple 5′ recombinant type infections were tested for significance using Student's *t*-test, and differences in the number of multiple 5′ recombinant types per person (i.e., multiplicity) were tested for significance using a two-tailed Mann-Whitney U test. Pairs of polymorphic blocks 2, 4a, 4b, and 6 and polymorphic sites in block 17 were subjected to an R^2 test to assess linkage disequilibrium, as described elsewhere.[21] Non-informative pairs (frequency < 10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Significance of linkage disequilibrium was assessed using an m × n chi-square test and two-tailed Fisher's exact probability test. The haplotype diversity index (*h*) of *msp1* haplotypes was calculated using formula (8-5) of Nei[30] $h = \{n/(n-1)\}x\{1-\Sigma pi^2\}$ where p and i are the frequency and number of *msp1* haplotypes, respectively. Variance (*V*) of *h* was calculated using the formula $V = \{2/n(n-1)\}[2(n-2)\{\Sigma pi^3 - (\Sigma pi^2)^2\} + \Sigma pi^2 - (\Sigma pi^2)^2]$ that was modified from formula (8-12) of Nei[30] for a haploid genome. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Diversity and multiplicity of 5′ recombinant types (blocks 2–6). Our typing method can potentially differentiate between 24 distinct 5′ recombinant types. Table 1 lists the distribution of 5′ recombinant types of the Solomon Islands samples collected in 1995 and 1996 and the Thai samples, together with our previously published data for Solomon Islands samples collected in 1994[26] and samples from Vanuatu.[19] The number of distinct 5′ recombinant types found in the Solomon Islands samples was 9 (n = 6 in 1994 and n =

TABLE 1
Prevalence of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) 5′ recombinant types in the Solomon Islands in comparison with Thailand and Vanuatu*

5′ recombinant type	Solomon Islands (1994)† Area B	Solomon Islands (1995–1996)				Thailand	Vanuatu‡
		Area A	Area B	Area C	All		
#1 KKKK	36	9	7	5	21	37	0
#2 MKKK	0	1	0	0	1	79	0
#3 RKKK	0	0	0	0	0	1	0
#13 KKKM	0	0	1	0	1	1	0
#14 MKKM	0	0	0	0	0	1	0
#15 RKKM	0	0	0	0	0	4	0
#16 KMKM	2	0	0	0	0	9	13
#17 MMKM	6	5	17	8	30	89	0
#18 RMKM	17	4	4	2	10	34	6
#20 MKMM	0	0	0	0	0	6	0
#21 RKMM	0	1	1	2	4	43	0
#22 KMMM	0	0	0	0	0	57	0
#23 MMMM	38	11	5	6	22	24	20
#24 RMMM	46	13	5	9	27	1	103
Total	145	44	40	32	116	386	142
Number of 5′ recombinant types	6	7	7	6	8	14	4
Rate of multiple infection (%)	51/84 (60.7)	8/35 (22.9)	11/15 (44.0)	10/22 (45.5)	29/82 (35.4)	103/107 (96.3)	1/141 (0.7)
Mean number of 5′ recombinant types	1.73	1.26	1.60	1.40	1.41	3.6	1.00–1.03§

* 5′ recombinant types are defined as associations of allelic types in msp1 blocks 2, 4a, 4b, and 6. K, M, and R denote K1, MAD20, and RO33 allelic types, respectively. Ten 5′ recombinant types were not identified in any of the three geographic areas: #4, KMKK; #5, MMKK; #6, RMKK; #7, KKMK; #8, MKMK; #9, RKMK; #10, KMMK; #11, MMMK; #12, RMMK; #19, KKMM.
† Sakihama and others.[26]
‡ Sakihama and others.[19]
§ Range of values separately calculated for the four islands of Vanuatu studied.

8 in 1995–1996), which is intermediate between the numbers obtained from Thailand (n = 14) and Vanuatu (n = 4). Distribution of 5′ recombinant types differed greatly among the three geographic areas (Table 1) ($P < 10^{-10}$ for all pairs compared). These differences are due mainly to sharp contrasts in frequencies of some 5′ recombinant types; e.g., the frequency of type 2 was 0.205, 0–0.023 and 0 in Thailand, the Solomon Islands, and Vanuatu, respectively, and the frequency of type 24 was 0.003, 0.125–0.317, and 0.725 in Thailand, the Solomon Islands, and Vanuatu, respectively. Five 5′ recombinant types (#3, #14, #15, #20, and #22) were found only in Thailand. The prevalence of infections with multiple 5′ recombinant types varied considerably among the three geographic areas (Table 1). The rate of multiple 5′ recombinant type infections in the Solomon Islands was 23–61%, which is intermediate between Thailand (96%) and Vanuatu (1%). All differences between pairs of geographic areas were significant ($P < 0.001$). The mean number of 5′ recombinant types per person (i.e., multiplicity) in the Solomon Islands (1.4–1.7) was also intermediate between Thailand (3.6) and Vanuatu (1.0). The differences in multiplicity were highly significant ($P < 10^{-4}$ for all pairs).

In contrast to these results, distribution of 5′ recombinant types did not differ significantly among areas A, B and C in Guadalcanal (Table 1), but differed significantly between samples collected in 1994 and samples in 1995 and 1996 in area B ($P < 0.0005$). This difference was due mainly to temporal fluctuations of five major 5′ recombinant types (#1, #17, #18, #23, and #24) (Table 1). A slight variation in the prevalence of multiple 5′ recombinant type infections was observed. In the samples collected in 1995 and 1996, the rate of multiple infections and multiplicity were higher in rural areas (areas B and C) than in Honiara City: multiple infection rate = 44–46% versus 23%, respectively; multiplicity = 1.4–1.6 versus 1.26, respectively. However, these differences were not significant. In area B, there was temporal variation in multiplicity between the samples from 1994 and the samples from 1995 and 1996 (1.7 versus 1.4, respectively), but the difference was not significant.

We examined effects of transmission season, host age, and parasite density on *msp1* diversity for samples (n = 35) collected in area A (Honiara) in 1995 and 1996. The rate of multiple infection and multiplicity were slightly higher in the rainy season (n = 11) than in the dry season (n = 24): multiple infection rate = 27.3% versus 20.8%, respectively; multiplicity = 1.36 versus 1.25, respectively. However, these differences were not significant. The multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years of age (n = 15 and 20, respectively). There was no difference in multiple infection rate or multiplicity between a parasite density of at least 4,000 parasites/μL and a parasite density less than 4,000 parasites/μL (n = 17 and 18, respectively). Similar analysis was not performed for populations in areas B and C because of limited or biased distribution of samples among seasons, ages, and parasite densities. In samples from area B collected in January 1994 (rainy season), the multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years old (n = 57 and 27, respectively).

3′ sequence types (block 17). The Solomon Islands samples contained all five previously reported nucleotide polymorphisms;[21,28] and their sequence types were Q-KNG-L, Q-KNG-F, E-KNG-L, and E-TSR-L (Table 2). All of these sequence types were also found in both Thailand and Vanuatu. Distribution of these 3′ sequence types differed greatly between the Solomon Islands and other areas: $P < 10^{-6}$ and $P < 10^{-10}$ for comparison with Thailand and Vanuatu, respectively. In Guadalcanal, there was no significant difference in distribution of 3′ sequence types among areas A, B, and C or between samples collected in 1994 and samples in 1995 and 1996. However, when samples were pooled to make two major groups for the Fisher's exact test, a significant difference appeared between 1994 and 1995–1996 ($P = 0.01$).

Diversity of *msp1* haplotypes. Among the Solomon Islands samples, there were six distinct *msp1* haplotypes in the 1994 samples and eight distinct *msp1* haplotypes in the 1995–1996 samples (Table 2). These numbers are comparable to those for Vanuatu (n = 6), but are much lower than those for Thailand (n = 16). Haplotype diversity (h) of the Solomon Islands populations (0.78–0.80) was intermediate between those of Thailand (0.89) and Vanuatu (0.43–0.68). There were marked differences in distribution of *msp1* haplotypes among the three geographic areas ($P < 10^{-10}$) (Figure 3). Consistently, of 19 distinct *msp1* haplotypes observed in these areas, only one haplotype (MMMM-ETSRL) was found in all areas. The Solomon Islands populations shared four haplotypes with Thailand and three haplotypes with Vanuatu. Only one haplotype was shared by Thailand and Vanuatu. These distributions suggest that the Solomon Islands populations comprise a transitional mixture between those of Thailand and Vanuatu.

In Guadalcanal, distribution of *msp1* haplotypes did not significantly differ among areas A, B, and C, but differed significantly between the 1994 samples and the 1995–1996 samples. This temporal difference is due largely to fluctuations in frequencies of *msp1* haplotypes in the two populations (Figure 3), similar to temporal fluctuations observed for frequencies of some 5′ recombinant types (Table 1).

Linkage disequilibrium in *msp1*. We analyzed linkage disequilibrium for the Solomon Islands area A 1995–1996 samples (n = 27) and area B 1994 samples (n = 30). Of the 15 informative pairs within and between polymorphic blocks 2–6 and polymorphic nucleotides in block 17, only one pair was not significant for both the Solomon Islands 1994 samples and 1995–1996 samples (Figure 4), which indicated strong linkage disequilibrium throughout the entire *msp1* gene. In contrast, the Thai population (n = 33) showed limited linkage disequilibrium: 7 of 15 pairs were not significant.

Polymorphism in *pfcrt*. All of the Solomon Islands isolates (n = 77) had an allele coding for SVMNT at residues 72–76 in the second exon of *pfcrt*; this is a Papua New Guinea-type chloroquine-resistant allele of *pfcrt*.[23] Recently, we reported the monomorphic prevalence of this resistant type in all four islands in Vanuatu,[31] suggesting monomorphic prevalence of the Papua New Guinea-type chloroquine-resistant *pfcrt* allele in the southwestern Pacific.

DISCUSSION

Several genetic mechanisms are involved in the generation of allelic diversity of *P. falciparum* genes: a point mutation causing a single nucleotide polymorphism; an insertion/

TABLE 2

Distribution of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) haplotypes in the Solomon Islands in comparison with Thailand and Vanuatu*

5' recombinant type	3' sequence type					No. of msp1 haplotypes	$h \pm SE$
	QKNGL	QKNGF	EKNGL	ETSRL	Total		
Solomon Islands (1994)							
KKKK	10	0	0	0	10		
MMKM	0	0	0	1	1		
RMKM	0	0	0	5	5		
MMMM	0	0	9	1	10		
RMMM	0	0	0	4	4		
Total	10	0	9	11	30	6	0.78 ± 0.04
Solomon Islands (1995–1996)							
KKKK	9	0	0	0	9		
MMKM	1	0	0	13	14		
RMKM	0	0	0	1	1		
MMMM	0	1	5	3	9		
RMMM	0	0	0	14	14		
Total	10	1	5	31	47	8	0.80 ± 0.03
Thailand							
KKKK	2	0	0	0	2		
MKKK	9	0	4	0	13		
KMKM	0	0	0	1	1		
MMKM	0	2	12	2	16		
RMKM	0	0	3	0	3		
RKMM	0	0	3	1†	4		
KMMM	1	1	2	3	7		
MMMM	0	0	1	1	2		
Total	12	3	25	8	48	16	0.89 ± 0.03
Vanuatu‡							
KMKM	0	0	13	0	13		
RMKM	0	0	0	6	6		
MMMM	0	0	0	19	19		
RMMM	0	69	6	27	102		
Total	0	69	19	52	140	6	0.43 ± 0.90–0.68 ± 0.07§

* *mspl* haplotype is defined as an association between 5' recombinant type and 3' sequence type. See Materials and methods for details of 5' recombinant types and 3' sequence type.
† One isolate with ETSRL type.
‡ Sakihama and others.[19]
§ Range of *h* separately calculated for the four islands of Vanuatu studied.

deletion of repetitive sequences, resulting in a repeat-length polymorphism; and meiotic recombination that produces novel alleles in progeny that differ from the parental alleles. Repeat length polymorphism is a prominent feature of *P. falciparum* antigen genes such as *mspl*, *msp2*, and glurp. Accordingly, repeat sequences in those loci have been widely used as polymorphic markers for genotyping of *P. falciparum*.[32] Repeat length polymorphism is probably generated by a mitotic replication slippage mechanism (slipped strand mispairing),[33] and is presumed to evolve rapidly.[26,31] Unequal crossing-over or gene conversion at meiosis may also be involved in the generation of repeat length polymorphism. Diversity of *mspl* haplotypes examined in the present study is distinct from that of repeat length polymorphisms, and is presumably generated by meiotic recombination in non-repetitive sequences. The present method identifies recombinant types in the 5' region (block 2–6), sequence types in the 3' regions (block 17), and *mspl* haplotypes (unique associations of 5' recombinant types and 3' sequence types). Simultaneously, this method monitors the number and distribution of 5' recombinant types in a parasite population, and multiplicity of infections (the number of 5' recombinant types per person). In the present study, we analyzed recombination-generated diversity of *mspl* and multiplicity of infections in a hyperendemic area in Guadalcanal, the Solomon Islands, and compared them with those of mesoendemic areas in Thailand and Vanuatu. We selected these geographic areas because all analysis of samples from these areas was performed under the same experimental conditions.

The diversity of 5' recombinant types and multiplicity of infections in Guadalcanal *P. falciparum* populations were lower than those of northwestern Thailand, despite the high endemicity in Guadalcanal. It is generally believed that diversity and multiplicity of infection increase as transmission frequency increases. Therefore, the present results are not consistent with this presumed correlation. A similar discrepancy has been reported in Papua New Guinea, where malaria transmission is more intense than in Thailand but the diversity of *msp2* and multiplicity of infections are relatively low.[34] The present results also indicate that linkage disequilibrium within *mspl* is greater in Guadalcanal than in Thailand. Linkage disequilibrium may derive from clustering of particular alleles. To avoid potential bias due to such clustering, we selected samples only from a local population (area A 1995–1996 samples and area B 1994 samples) and did not combine populations from different areas of Guadalcanal. Nevertheless, we detected strong linkage disequilibrium in *mspl*, indicating that limited recombination events are the most likely cause of this linkage disequilibrium. Although the annual entomologic inoculation rates (EIRs) of the present study areas were not available, previous reports indicate that areas close to area B have a very high EIR: 584–1,022 bites per person per year,[35] a level comparable with those of high-transmission

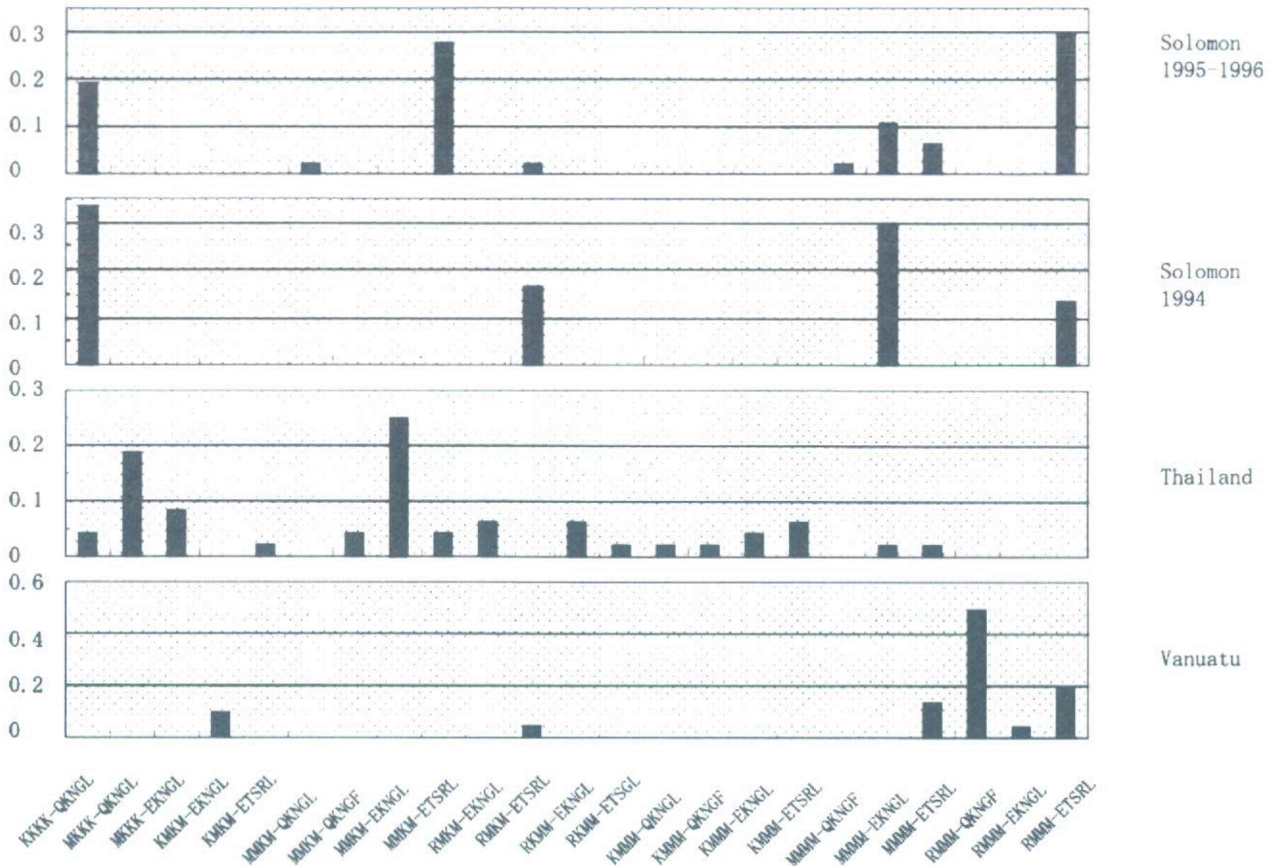

FIGURE 3. Frequency distribution of merozoite surface protein 1 (*msp1*) gene haplotypes in *Plasmodium falciparum* isolates from the Solomon Islands, Thailand, and Vanuatu. The *msp1* haplotypes, which are unique associations of 5′ recombinant types and 3′ sequence types, are indicated along the x-axis.

areas in Africa such as Tanzania.[36] Thus, the question arises as to why linkage disequilibrium is strong in a high-transmission area. We have previously proposed that frequency of transmission is not the sole determinant of the strength of linkage disequilibrium in *msp1*, and that other factors are also intimately involved.[19] We suggest that at least three variables are associated with linkage disequilibrium in *msp1*: 1) the number of alleles prevalent in a local area, 2) the rate of multiple allele infections, and 3) the number of alleles per infection (multiplicity). Effects of those variables were weak or relatively limited in the present samples from Guadalcanal, compared with the samples from Thailand. This suggests a low frequency of out-crossing, resulting in the observed linkage disequilibrium in Guadalcanal. In addition to the three variables, limited gametocyte production among the asexual parasite populations may be added: infections in Guadalcanal are in some way synchronized, so that gametocyte production at any one time may be limited to one of the asexual clones present, whereas in Thailand this is not the case.

Since epidemiologic settings vary substantially between and within geographic areas, a direct comparison of multiplicity of infection must be cautious. Multiplicity tends to decrease with age in areas highly endemic for malaria and it is lower in asymptomatic carriers than in individuals with clinical malaria.[5,37] Age-dependent acquisition of strain-specific immunity, which is mounted after repeated infections of different genotypes, is considered to contribute to the reduction of multiplicity. In contrast, in low-transmission areas multiplicity does not always correlate with age or the presence of clinical malaria. In the present study, isolates were collected from both symptomatic patients and asymptomatic carriers. We were unable to find a relative reduction of multiplicity of *msp1* 5′ recombinant types in asymptomatic partially-immune adults in rural areas (areas B and C) in Guadalcanal because our samples from rural villages were limited in number and had biased distribution among ages (primarily school children) and parasite density. We therefore do not consider that our results exclude the presence of age-dependent reduction of multiplicity in asymptomatic semi-immune carriers in the Solomon Islands. Nevertheless, multiplicity in area A (Honiara City) was significantly lower than in areas B and C. Acquired strain-specific immunity might not be so intense to significantly reduce multiplicity in areas B and C. Low multiplicity in area A, compared with areas B and C, may simply be due to limited mosquito biting or relatively easy accessibility to anti-malarial drugs. Our Thai samples were from clinical cases, and thus are comparable with hospital samples (area A) in Guadalcanal. Multiplicity in Thailand was significantly higher than in Guadalcanal, despite a lower transmission level in Thailand. Samples from Vanuatu were from asymptomatic carriers and thus comparable to those from

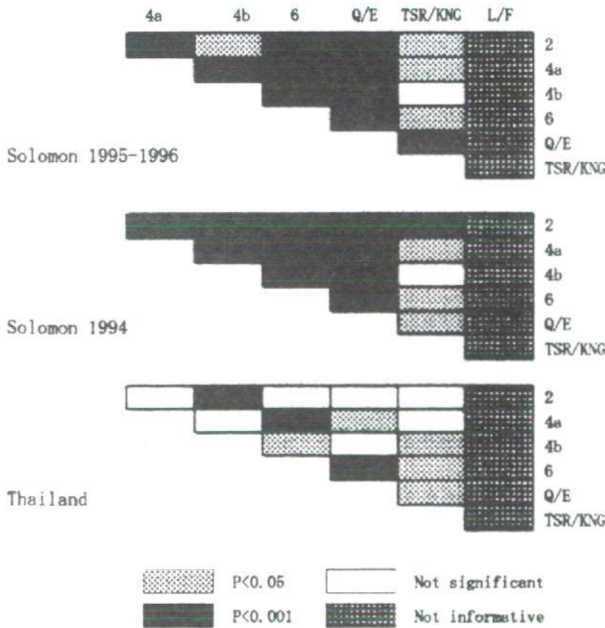

FIGURE 4. Linkage disequilibrium in the *Plasmodium falciparum* merozoite surface protein 1 gene in populations from the Solomon Islands (area A = 1995–1996; area B = 1994) and Thailand. Pairs of polymorphic blocks 2, 4a, 4b, and 6, and three polymorphic residue sites (Q/E, TSR/KNG, and L/F) in block 17 were subjected to the R^2 test.

rural villages in Guadalcanal (areas B and C). Multiplicity in Vanuatu was nearly 1.0 and significantly lower than in Guadalcanal, where transmission is more stable and higher than in Vanuatu. These results indicate that the level of multiplicity in the Solomon Islands was intermediate between Thailand and Vanuatu, reinforcing the idea that multiplicity is not always associated with the intensity of malaria transmission.

The present study also showed temporal variation in the distribution of *msp1* haplotypes in area B in Guadalcanal between the 1994 samples and the 1995–1996 samples. The present finding that all isolates from the two populations have the chloroquine-resistant *pfcrt* alleles indicates that a population change due to chloroquine pressure is not responsible for this temporal fluctuation. A possible explanation for this fluctuation is that the area B 1994 samples and 1995–1996 samples were from different villages. However, for both the 1994 samples and 1995–1996 samples, we found no difference in distribution of *msp1* haplotypes among villages. Also, within a single village (Tadhimboko in area B), the distribution differed significantly between 1994 samples and 1995–1996 samples. Thus, there was a temporal change in distribution within area B. A previous study has shown considerable temporal variation in distribution of *msp1* 5′ recombinant types in hypoendemic areas in Brazil.[38] The temporal variation in Brazil may be due to migration of laborers. In addition to such migration, strain-specific immunity to certain *msp1* haplotypes may be involved in the temporal variation presently observed in the distribution in the Solomon Islands. Studies of *P. falciparum* populations from Irian Jaya suggest that strain-specific immunity is a factor in temporal variation in frequency of *msp2* alleles.[39]

In the present study, Guadalcanal *P. falciparum* populations did not exhibit seasonal change in the distribution of *msp1* 5′ recombinant types, the rate of multiple infections, or multiplicity of infections. A clear seasonal variation in the diversity of antigen genes and multiplicity has been observed in low-transmission areas such as Sudan,[40] where transmission ceases nearly completely during the dry season. In the Solomon Islands, transmission of malaria is perennial, although it decreases during the dry season. Therefore, a direct comparison of seasonal changes in transmission cannot be made between the Solomon Islands and Sudan. In a perennial transmission area in Benin, reduced transmission had no substantial influence on the diversity of *msp2* alleles or multiplicity of infections.[41] Thus, seasonal changes in malaria transmission do not always affect the diversity of *msp1* alleles and multiplicity of infections in area with relatively high transmission.

The present study is the first to document the prevalence of particular *pfcrt* alleles in the Solomon Islands. *Plasmodium falciparum* populations in northern Guadalcanal exhibited monomorphic prevalence of a Papua New Guinea type of chloroquine-resistant *pfcrt* in both 1994 samples and 1995–1996 samples. Chloroquine is the mainstay for treatment of malaria and is effective against *P. vivax* malaria in the Solomon Islands[24] and Vanuatu.[42] Thus, the persistence of chloroquine pressure may be the cause of the monomorphic prevalence of chloroquine-resistant *pfcrt* in the southwestern Pacific.

In conclusion, the present study presents evidence that allelic diversity of *P. falciparum msp1* as measured by *msp1* haplotypes is not entirely dependent on the intensity of transmission. Populations from the Solomon Islands had significantly lower diversity compared with that from Thailand with a lower level of transmission. Linkage disequilibrium in *msp1* was also significantly higher in the Solomon Islands compared with Thailand. These findings indicate that frequency of recombination events in *msp1* is determined not only by transmission intensity but also by the number of *msp1* alleles prevalent in an area and multiplicity of infections.

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2-Cys Peroxiredoxin TPx-1 is involved in gametocyte development in *Plasmodium berghei*

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Abstract

Peroxiredoxins (Prxs) constitute a ubiquitous family of antioxidant enzymes involved in diverse cellular functions including cell proliferation and differentiation. To investigate the physiologic role of typical 2-Cys Prx in malaria parasites (TPx-1), we disrupted this gene in the rodent malaria parasite *Plasmodium berghei* (*pbtpx-1*). The gene-disrupted parasite (Prx KO) developed normally in mouse erythrocytes and multiplied at a rate similar to that of the parent strain (WT) during the experimental period. The normal growth rate was not altered after 10 passages, and the level of 8-hydroxy-2′-deoxyguanosine, which accumulates in the parasite genome during the cell cycle, was similar between Prx KO and WT. These results suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx, and that it is not essential for asexual parasite growth in mouse erythrocytes. However, Prx KO produced up to 60% fewer gametocytes, sexual-stage parasites involved in the transition between the mammalian host and the mosquito, than WT did. The peak of gametocytemia was also delayed; however, the male/female ratio of gametocytes and the exflagellation activity of male gametocytes were normal. These results suggest that TPx-1 is required for normal gametocyte development but does not affect the male/female gametocyte ratio or male gametogenesis. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, these findings suggest, for the first time, the involvement of Prx in the sexual development of the malaria parasite.
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Keywords: Gametocyte; Peroxiredoxin; *Plasmodium berghei*; Thioredoxin peroxidase

1. Introduction

As *Plasmodium* spp. actively proliferate within erythrocytes of their vertebrate hosts, large quantities of reactive oxygen species (ROS), which damage biologic macromolecules, are generated [1,2]. A major source of ROS in parasite cells is heme, a byproduct of hemoglobin digestion for amino acid procurement [3,4]. ROS are also generated when the organism is exposed to various stress conditions such as that induced by the host immune system [5]. *Plasmodium* spp. (the malaria parasites) are highly susceptible to oxidative stress, and their antioxidant defenses are considered to play essential roles in their asexual development. They are thus potential targets for chemotherapy [6,7].

To protect biologic macromolecules from the effects of ROS, aerobes have evolved efficient defense systems composed of nonenzymatic and enzymatic antioxidants [8]. The four major cellular antioxidant enzymes are superoxide dismutase, catalase, glutathione (GSH) peroxidase, and peroxiredoxin [9]. Peroxiredoxins (Prxs) constitute a family of proteins structurally homologous to the thiol-specific antioxidant of yeast [10] and have been identified in all living organisms, from bacteria to human [11,12]. There are three subtypes of Prxs, 1-Cys Prx, typical 2-Cys Prx, and atypical 2-Cys Prx. Although the cellular function of 1-Cys Prx and electron donor for the molecule remain controversial [12,13], 2-Cys Prxs have been found to act

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GSH, glutathione; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Prx, peroxiredoxin; ROS, reactive oxygen species

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as a terminal peroxidase that reduces hydrogen peroxide and organic hydroperoxides with the use of electrons donated by the thioredoxin (Trx) system [11,12]. With respect to the cellular functions of 2-Cys Prx, diverse findings have been reported in mammals, including modulation of cytokine-induced hydrogen peroxide levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation, and apoptosis [9,12,14].

Malaria parasites do not possess catalase or genuine GSH peroxidase in their genome, but the parasites are equipped with a 1-Cys Prx and two typical 2-Cys Prxs [1,2]. Thus, it is believed that GSH itself is the major redox buffer for transient H_2O_2 exposure and that the basal cellular peroxide flux is dealt with by the Trx system, which includes Prxs [7]. The 1-Cys Prx and one of the 2-Cys Prxs are expressed in the cytosol, and the other 2-Cys Prx is localized in mitochondria [15]. We recently reported that disruption of the gene encoding the cytosolic 2-Cys Prx (PlasmoDB; PF14_0368; PfTPx-1) in *P. falciparum* renders parasites hypersensitive to ROS and reactive nitrogen species, although it did not affect parasite growth under normal culture conditions [16]. This suggests that this Prx is not essential for parasite survival under culture conditions and that physiologic function of Prx in parasite cells remains to be elucidated. This Prx is constitutively expressed during the asexual development as well as in the gametocyte both in *P. falciparum* [17] and in the rodent malaria parasites [18,19]. To determine the cellular function of 2-Cys Prx in malaria parasites, we disrupted the gene encoding TPx-1 in the rodent malaria parasite *P. berghei* and analyzed the phenotype of the gene-disrupted parasite in infected mice. The disruptant showed normal asexual proliferation in mouse erythrocytes but showed a defect in gametocyte development, the sexual stage for transition to mosquitos that is induced by host environmental factors.

2. Material and methods

2.1. Parasites

The *P. berghei* ANKA strain was obtained from the Armed Forces Research Institute of Medical Sciences, Thailand. The parasite was maintained by mosquito transmission in *Anopheles stephensi* interspersed by a maximum of two serial passages in DBA/2 or Balb/c mice (Clea Japan, Tokyo, Japan). The animal experiments in this study were carried out in compliance with the Guide for Animal Experimentation of the International Medical Center of Japan.

2.2. Plasmid construction

For disruption of the PbTPx-1 gene, 5′ and 3′ portions of the gene were amplified by polymerase chain reaction (PCR) and cloned into the targeting vector pMD204 [20], which was supplied by the Malaria Research and Reference Reagent Resource Center; MR4/ATCC, Manassas, VA, USA. Each fragment contained part of the coding sequence and flanking region was amplified with sequence-specific primers and parasite genomic DNA. The primers used for the 5′ fragment were 5′-GG GGG CCC TCA CCA GCC TTA TTA AG-3′ and 5′-CC CCC GTC GACAAT ATA TTT CTT TCC-3′ (*Apa*I and *Hinc*II sites are underlined). The primers used for the 3′ fragment were 5′-CG GAA TTC CGA GTT TGT AAA AGA AC-3′ and 5′-TT CTG CAG TCA TTT AAA ATA AAG-3′ (*Eco*RI and *Pst*I sites are underlined). The primers were designed on the basis of sequences in the *P. berghei* genome database provided by the *Plasmodium* Genome Resource (PlasmoDB; PB_5804.1 and PB000037.01.0). PCR products were purified and cloned into upstream or downstream of the pyrimethamine-resistant form of *P. berghei* dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene, which was used as a selectable marker. For gene targeting experiments, the plasmid was digested with *Apa*I and *Pst*I to separate the linear targeting construct from the plasmid backbone.

2.3. Transfection and selection of disruptant

Transfection and selection were performed essentially according to the protocols of Ménard and Janse [21]. Blood-stage parasites used for transfection were prepared from Jcl:Wistar rats (Clea Japan) that had been infected by intraperitoneal (i.p.) injection of 5×10^6 parasitized erythrocytes per animal. Animals were killed when parasitemia reached 3%. Blood was drawn from the animal into heparinized syringes (100 μg/ml of blood) by cardiac puncture under ether anesthesia. Samples were washed for 10 min at $160 \times g$ at room temperature (RT) with RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 25 mM HEPES and 20% heat-inactivated fetal calf serum. The packed cells were then resuspended in 40–50 ml (10 volumes of the blood sample) of the medium and cultured in glass petri dishes (20–25 ml of the blood suspension/dish) in a AnaeroPack® microaerophilic culture system, which provided 5% CO_2 and 8% O_2 gases condition (Mitsubishi Gas Chemical, Tokyo, Japan), for 16 h at 37 °C with gentle rotation (50 rpm). Blood suspensions in the dishes were then layered onto 7.5 ml Nycoprep™ density gradient 1.077 medium (Axis-Shield, Oslo, Norway) and centrifuged first at $160 \times g$ for 5 min and then at $360 \times g$ (by shifting to high speed before stop) for 15 min at RT. Schizonts at the interface were collected and washed with 40 ml culture medium for 5 min at $360 \times g$ at RT and then with 3 ml phosphate-buffered saline (PBS). Schizont pellets were resuspended in 400 μl PBS containing 50 μg linearized targeting vector, then transferred to 0.4 cm electroporation cuvettes for transfection. Mixtures were subjected to an electric pulse (800 V, 25 μF) with a Gene Pulser® II System (Bio-Rad, Hercules, CA, USA) and were immediately inoculated into two rats (200 μl electroporated schizont suspension/animal) by intravenous (i.v.) injection. Animals were treated with pyrimethamine (12.5 mg/kg body weight) at 30 h after inoculation every 12 h until parasites were no longer detected in the blood circulation. When the parasitemia increased again to 1–2%, rats were treated with the same dose of pyrimethamine, and resistant parasites were transferred to new rats in which the parasites multiplied in the absence of drug. When parasitemia had reached 1%, the parasite population in

each rat was separated into wild-type and disruptant by limiting dilution. Both parasite genomes were determined by PCR and Southern blot analysis.

2.4. Nucleic acid techniques

Parasite-infected blood was passed through a CF11 (Whatman, Maidstone, Kent, UK) column and span with Lymphoprep™ density gradient medium (Axis-Shield, Oslo, Norway) to remove leukocytes. Parasite-infected erythrocytes were then washed with PBS and lysed with PBS containing 0.05% saponin. Parasite pellets were washed several times with PBS, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Nucleic acids were extracted from parasite pellets. For Southern blot, genomic DNA was extracted by the standard phenol–chloroform method and incubated with *Spe*I. For Northern blot, total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Nucleic acids were separated by agarose gel electrophoresis and transferred onto HyBond N^+ membranes (Amersham Biosciences UK Ltd., Little Chalfont, UK). Membranes were probed with a 368-bp fragment spanning the 5′ sequence of *pbtpx-1* for Southern blot (Fig. 1B) and the entire coding sequence for Northern blot (Fig. 2B). The probes were labeled, hybridized, and detected with the AlkPhos Direct Labeling and Detection System (Amersham Biosciences).

2.5. Western blot analysis and indirect immunofluorescence assay

For Western blot analysis, parasite pellets were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 5% 2-mercaptoethanol [22]. After separation by SDS-PAGE (12.5%), parasite proteins were transferred electrophoretically to polyvinylidene difluoride sheets (Immobilon; Millipore, Bedford, MA, USA) and incubated with anti-recombinant PfTPx-1 (rPfTPx-1) rabbit IgG (25 μg/ml). Interspecific crossreactivity between PbTPx-1 and anti-rPfTPx-1 rabbit IgG has been reported [18]. Immune complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH, USA). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Indirect immunofluorescence assay was performed as described previously [15]. The IgG fraction of rabbit antisera to rPfTPx-1 was used at 20 μg/ml. Parasite cells on slides were mounted with Prolong AntiFade Solution (Molecular Probes, Eugene, OR, USA) and observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

2.6. Analysis of parasite development in mice

Five-week-old female Balb/c and C57B6 mice (Clea Japan) were infected with *P. berghei* (10^6 parasitized cells per mouse) by i.p. injection. Parasitemia and gametocytemia were determined by microscopic examination of Giemsa-stained thin blood films. Male and female gametocytes were distinguished by size and coloration. Numbers of exflagellation centers were counted according to a previously described method [23]. Accumulation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the parasite genome was evaluated with a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan) [24]. DNA for ELISA was extracted from parasites collected from infected mice (blood from 10 infected mice was pooled) when

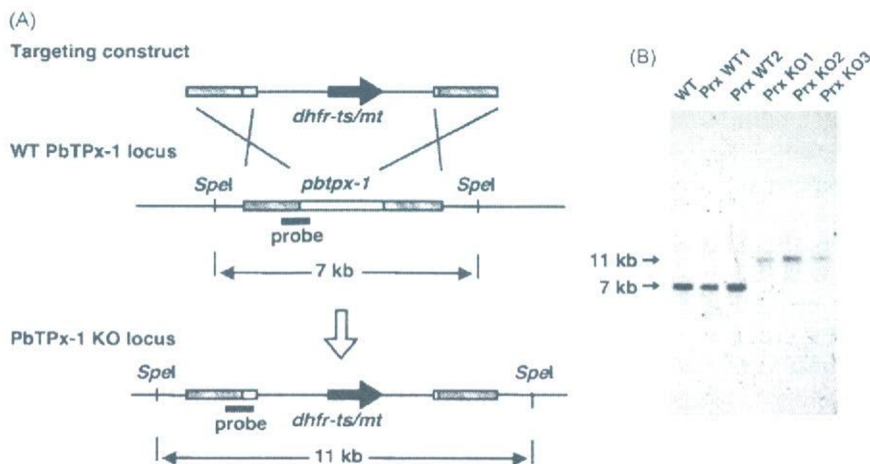

Fig. 1. Targeted disruption of the TPx-1 gene in *P. berghei*. (A) The targeting construct, composed of the 5′ and 3′ regions (hatched boxes) and partial open reading frame (open box) of the PbTPx-1 gene (*pbtpx-1*) and the pyrimethamine-resistant variant of DHFR-TS gene of *P. berghei* (*dhfr-ts/mt*) as a selectable marker (filled arrow), was integrated into the wild-type (WT) PbTPx-1 locus by double-crossover homologous recombination. Recombination disrupts *pbtpx-1* and creates the locus containing *dhfr-ts/mt* (PbTPx-1 KO locus), which confers pyrimethamine resistance to disruptants. The *Spe*I restriction sites and the position of the probe used for Southern blot analysis in B are indicated. (B) Southern blot analysis of genomic DNA samples from the parent parasite (WT), two wild-type parasite populations with pyrimethamine resistance (*dhfr-ts/mt* at the DHFR-TS locus) (Prx WT), and three *pbtpx-1* disruptant populations (Prx KO). DNA samples were digested with *Spe*I, separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized with probe. The single 11-kb band in the Prx KO populations indicates *pbtpx-1* disruption, whereas the single 7-kb band in the WT and Prx WT populations indicates an intact PbTPx-1 locus.