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Worldwide sequence conservation of transmission-blocking vaccine candidate Pvs230 in *Plasmodium vivax*

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ABSTRACT

Pfs230, surface protein of gametocyte/gamete of the human malaria parasite, *Plasmodium falciparum*, is a prime candidate of malaria transmission-blocking vaccine. *Plasmodium vivax* has an ortholog of Pfs230 (Pvs230), however, there has been no study in any aspects on Pvs230 to date. To investigate whether Pvs230 can be a vivax malaria transmission-blocking vaccine, we performed evolutionary and population genetic analysis of the Pvs230 gene (*pvs230*: PVX_003905). Our analysis of Pvs230 and its orthologs in eight *Plasmodium* species revealed two distinctive parts: an interspecies variable part (IVP) containing species-specific oligopeptide repeats at the N-terminus and a 7.5 kb interspecies conserved part (ICP) containing 14 cysteine-rich domains. Pvs230 was closely related to its orthologs, Pks230 and Pcys230, in monkey malaria parasites. Analysis of 113 *pvs230* sequences obtained from worldwide, showed that nucleotide diversity is remarkably low in the non-repeat 8-kb region of *pvs230* ($\theta\pi = 0.00118$) with 77 polymorphic nucleotide sites, 40 of which results in amino acid replacements. A signature of purifying selection but not of balancing selection was seen on *pvs230*. Functional and/or structural constraints may limit the level of polymorphism in *pvs230*. The observed limited polymorphism in *pvs230* should ground for utilization of Pvs230 as an effective transmission-blocking vaccine.

1. Introduction

Malaria is a major infectious disease caused by protozoa of the genus *Plasmodium* and transmitted by anopheline mosquito. There were an estimated 243 million clinical cases and 863,000 malaria-related deaths in 2008 [1]. Among four species of human malaria parasites, *Plasmodium vivax* was the most globally distributed. Although often malaria caused by *P. vivax* is regarded as a benign and self-limiting infection, there is an increasing evidence that the overall burden, the economic impact, and the severity of

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disease associated with *P. vivax* have been underestimated. Particularly in Asia and Pacific, many endemic countries now wish to eliminate *P. vivax* malaria totally. The elimination strategies are however limited and confounded by emergence of multidrug resistant isolates and relapse from dormant hypnozoites stages at varying time intervals after the initial infection and there have not been any available optimal chemotherapeutic agents to combat against these situations [2,3]. Therefore, the development of vaccines against *P. vivax* is a necessary component towards malaria elimination [4]. Malaria vaccines are generally divided into three groups based on stages of the parasite life cycle that are targeted: pre-erythrocytic, asexual blood-stage, and transmission-blocking vaccines (TBVs). Pre-erythrocytic vaccines act against sporozoites and liver-stage parasites and are designed to prevent infection. Asexual blood-stage vaccines are aimed at reducing parasite multiplication and growth to protect against clinical symptoms, but not infection. TBVs are aimed at blocking malaria transmission by interrupting the parasite life cycle in the mosquito. TBVs as such do not directly protect vaccinated individuals from infection; however, they could contribute to elimination of the disease by lowering the parasite transmission efficiency.

TBVs elicit antibodies against surface antigens of sexual- and mosquito-stage parasites and, thus, arrest subsequent development of parasite in the mosquito midgut [5]. Target antigens for TBV development are sexual- and mosquito-stage specific surface molecules. Antigens specifically expressed by zygotes and ookinetes in the mosquito midgut (e.g., P25 and P28 in *P. falciparum* and *P. vivax*), referred to as post-fertilization target antigens, have been shown to be effective for inducing transmission-blocking immunity [6–8]. The ookinete surface antigens, Pfs25 in *P. falciparum* and Pvs25 in *P. vivax*, have been tested in Phase I clinical trials, and a positive correlation of the TBV efficacy with the antibody titer against each vaccine in the volunteers have been demonstrated [9–11]. However, P25 is not a boostable TBV candidate because it is not expressed by blood-stage parasites and hence it is not exposed to host immune response in natural infections [12]. In contrast to ookinete surface antigens, antigens that are involved in fertilization of male and female gametes, referred to as pre-fertilization target antigens, may be boostable TBV candidate antigens because they are also expressed in the gametocytes in the human blood and exposed to human immune response. Major proteins found on the surface of both male and female gametocytes/gametes such as Pfs48/45 and Pfs230 belong to a family defined by the presence of a unique arrangement of six cysteine-containing domains [13]. Pfs48/45, expressed on the surface of gametocytes/gametes, contains three cysteine rich domains (CRDs) [14,15]. Pfs230 is a 360-kDa surface protein, which is also expressed on the surface of gametocytes/gametes, contains 14 CRDs [16–19]. In *P. falciparum* the Pfs230 minus males have reduced ability to interact with erythrocytes and fewer oocysts are produced [20]. Monoclonal antibodies (mAbs) against the CRDs can potently be effective in blocking the infectivity of the parasites to mosquitoes [21–25]. Antibodies raised against a recombinant protein corresponding to a N-terminal 76-kDa part of the mature Pfs230 have been shown to reduce the ability of *P. falciparum* parasites to infect mosquitoes [26]. Importantly, Pfs230 elicits humoral immune responses in infected individuals that can mediate transmission-blocking immunity [27,28]. These accumulating evidence supports the priority of Pfs230 as candidate of falciparum TBV.

Most of the antigens expressed on the surface of asexual blood-stage parasites are highly polymorphic and under positive selection [29]. Polymorphic antigens are likely to induce immune responses in an allelic variant-dependent manner [30,31] and thus may limit the efficacy of vaccines based on such antigens. In contrast, antigens expressed in the sexual- and mosquito-stages show limited polymorphism; these include Pfs25 [9], Pfs28 [32], Pfs230 [17,33,34], Pvs25 and Pvs28 [35,36]. *P. vivax* has an ortholog of Pfs230 (Pvs230) [37]. Searching for immunodominant and conserved domains in this large molecule is of particular importance to design the vaccine antigen. There has been no study in any aspects on Pvs230 to date. We therefore have decided to investigate whether Pvs230 can be a promising TBV candidate. We performed evolutionary and population genetic analyses of the *pvs230*, specifically addressing (i) the evolutionary relatedness of *pvs230* with its orthologs in other seven *Plasmodium* species and (ii) nucleotide polymorphism of *pvs230* in *P. vivax* populations from diverse geographical areas. Comparative sequence analysis identified interspecies variable- and conserved-parts in *pvs230*, and provides evidence that Pvs230, particularly CRDs, has limited polymorphism. These results reinforce that Pvs230 can be a promising TBV candidate of *P. vivax*.

2. Materials and methods

2.1. Parasite isolates and DNA extraction

P. vivax isolates were collected from patients in seven endemic countries; Brazil, Turkey, Madagascar, China, Thailand, Papua New Guinea (PNG) and Solomon Islands. In Brazil, 22 isolates were collected from Acre state, northwestern Brazil, between 1999 and 2006 [38,39]; in Turkey, 20 isolates were collected from Siverek and Harran in Sanliurfa province, southeastern Turkey in June–November 2007–2008 [40]; in Madagascar, 16 isolates were collected from various regions (Sainte Marie in eastern coast, Taolagnaro in southern coast, Antananarivo and Ankazobe in central highland, Saharevo in eastern foothill, and Ampasimpotsy in western foothill) in 2000–2005; in China, 22 isolates were collected from Hubei, southeastern China in July to October 2000; in Thailand, 37 isolates were collected from Mae Kasa and Mae Sod in Tak Province, western Thailand in January 1999–October 2000; in PNG, 29 isolates were collected from Kiniambu and Jawia villages, Wewak, East Sepik Province in northeast coast in August–September 2001 [41]; in Solomon Islands, 18 isolates were collected from area B of northern Guadalcanal island in February 2007–2008 [42]. In all cases, ethical clearance for sampling was approved from relevant ethical committees, and informed consent was obtained from patients or their guardians. In Turkey, PNG and Solomon Islands, finger-prick blood was collected on Whatman® 31ETCHR filter paper. Parasite genomic DNA was extracted from filter blots using the EZ1 DNA Investigator kit on the EZ1 BioRobot™ (Qiagen, Germany). In other countries, parasite DNA was extracted from venous blood, using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Three *Aotus* monkey-adapted *P. vivax* isolates were additionally obtained from ATCC (American Type Culture Collection): Chesson strain (PNG) (ATCC #30060), Nicaragua strain (ATCC #30073) and Panama strain (ATCC #30138). Genomic DNA from *P. cynomolgi* (B strain) (ATCC #30129), a *P. vivax*-related monkey malaria parasite [43] was also used. Genomic DNA from *P. yoelii* (17XNL strain) was extracted from infected mouse blood, using QIAamp DNA Blood Mini Kit (QIAGEN).

2.2. DNA sequencing

The Pvs230 gene (*pvs230*) was amplified by PCR. PCR primers were designed from the *P. vivax* Sal-1 *pvs230* sequence (PlasmoDB, Gene ID PVX_003905; http://plasmodb.org/plasmo/) (Suppl. Table 1). Amplification was carried out in a 20 μl reaction mixture containing 0.2 μM each of forward and reverse primers, 400 μM each of dNTP, 1 unit of LA-Taq (Takara, Otsu, Japan), 2 μl of 10× PCR buffer, 2.5 mM of $MgCl_2$, and 1 μl of genomic DNA. PCR conditions were as follows: initial denaturation at 93 °C for 1 min, and

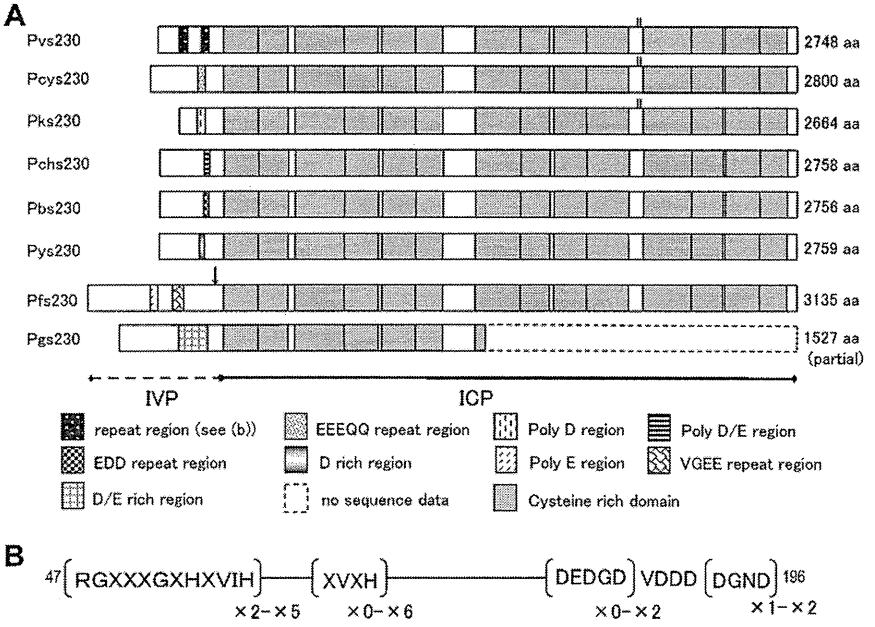

Fig. 1. Primary structure of Pvs230 and its orthologs. In (A) deduced amino acid sequences obtained from *P. vivax* (Pvs230), *P. cynomolgi* (Pcys230), *P. knowlesi* (Pks230), *P. chabaudi* (Pchs230), *P. berghei* (Pbs230), *P. yoelii* (Pys230), *P. falciparum* (Pfs230), and partial sequence of *P. gallinaceum* (Pgs230: aa 1–1527) are aligned. In interspecies variable part (IVP), tandem repeat regions are represented as variously marked boxes, and 14 cysteine-rich domains in interspecies conserved part (ICP) are represented as half-tone boxes. Predicted cleavage sites of Pfs230 are marked by an arrow. Two cysteine residues between CRD10 and CRD11 in Pvs230, Pcys230 and Pks230 are shown in small bars. Predicted amino acid sizes are shown in right of respective amino acid sequences. In (B) repeat motifs and their repeat number in Pvs230 are shown, in which X denotes any amino acid residues. Major repeat units in RGXXGXHVIH are RGSYEGIHQVIH, RGRCEGIHQVIH, and RGRCDGGHHVIH, those in XVXH are RVVH, CVVH, RVAH, and RVIH (Suppl. Fig. 2). Amino acid positions are numbered after the Pvs230 sequence of Sal-1 strain (GenBank accession #XM_001612970).

amplification for 40 cycles at 93 °C for 20 s and 62 °C for 4 min, followed by a final extension at 72 °C for 10 min in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR products were subjected to DNA sequencing using the Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems) in an Applied Biosystems 3130xl Genetic Analyzer. Sequencing primers were designed to cover target regions from both directions (Suppl. Table 1). Sequences from fifty-four samples showed superimposed electropherogram peaks, indicative of mixed genotype infections, and thus they were excluded from further analysis (Suppl. Table 2). Sequences were verified by at least two independent amplifications from the same DNA. We confirmed the Sal-1 *pvs230* sequence and used as a reference sequence. For those samples from PNG and Solomon Islands, in which PCR products were not sufficient for direct sequencing, the nested PCR was performed using internal primers (Suppl. Table 1). P230 sequence from a *P. vivax*-related monkey malaria parasite, *P. cynomolgi* (B strain) [43], *pcys230*, was also obtained by direct sequencing as described above, using specific primers (Suppl. Table 1). P230 gene sequence from a rodent malaria parasite, *P. yoelii* (17XNL strain), *pys230*, was also obtained as described above.

Sequences obtained in this study have been deposited to DDBJ/EMBL/GenBank under accession numbers AB574508-AB574621.

2.3. Sequence alignments and analyses

We obtained a total of 112 *pvs230* sequences from *P. vivax* isolates in seven countries (Suppl. Table 2). P230 sequences were also determined from *P. cynomolgi* (*pcys230*) and *P. yoelii* (*pys230*). P230 sequences from other malaria parasite species retrieved from PlasmoDB were *P. vivax* (*pvs230*; Gene ID PVX_003905), *P. falciparum* (*pfs230*; PFB0405w), *P. knowlesi* (*pks230*; PKH_041100) and *P. chabaudi* (*pchs230*; PCAS_030830). Additionally, *P. berghei* P230 (*pbs230*) sequence was obtained from Sanger Institute database. *P. gallinaceum* P230 partial sequence (Pgs230: aa 1–1527) was obtained by NCBI Blast search in *P. gallinaceum* genome, Pg_2265551.c000320551.Contig1. Sequences were aligned using CLUSTAL W [44] implemented in MEGA version 4 software [45] with manual corrections. A phylogenetic tree was constructed using the Neighbor-Joining method [46] with the Jukes and Cantor correction implemented in MEGA. Bootstrap values were obtained by 1000 heuristic replications.

Sequence polymorphism was estimated by the following parameters: (1) the number of polymorphic nucleotide sites (*S*), (2) the number of haplotypes (*h*), (3) haplotype diversity (Hd), and (4) the observed average number of pairwise nucleotide difference per site ($\theta\pi$). These estimates were calculated using the DnaSP v4.10.9 software [47]. To estimate the proportion of genetic variance due to population subdivision, the Wright's fixation index [48] of inter-population variance in allele frequencies, termed Fst, was calculated using the Arlequin software [49]. Overall Fst for all six parasite populations, in which more than seven sequences were available, and pairwise Fst were estimated.

2.4. Tests for departure from neutrality

In this study, we used the Nei and Gojobori method [50] with the Jukes and Cantor correction as implemented in the MEGA [45] to examine departure from neutrality. This method estimates the difference between the numbers of synonymous substitutions per synonymous site (dS) and of nonsynonymous substitutions per nonsynonymous site (dN). Standard error was determined by 1000 bootstrap replications, and dN and dS were compared with a *Z*-test using MEGA. If dN is significantly higher than dS, positive selection (diversifying selection) appears to be acting, while if dS is higher than dN, purifying selection is predicted.

We also used Tajima's *D* test, and McDonald–Kreitman (MK) test to examine departure from neutrality. Tajima's *D* test measures allele frequency spectrum by comparing $\theta\pi$ and θs, the latter

of which is the standardized number of polymorphic sites per site [51]. Under neutrality a value of Tajima's D is expected to be 0. Significantly positive values of Tajima's D suggest recent population bottleneck or balancing selection, whereas negative values suggest population growth or directional selection. Fu and Li's D^* and F^* tests were also used to test for excess or lack of singleton nucleotides by comparing estimates of θs based on the number of singletons vs. that derived from S (the D^* index) or $\theta\pi$ (the F^* index) [52]. All of these estimates were calculated using DnaSP. The MK test [53] was used to assess a signature for selection, in which the ratio of nonsynonymous to synonymous substitutions was compared between polymorphic difference (within species) and fixed difference (between closely related species) using DnaSP. Under neutrality, these ratios will be similar, whereas an excess of intraspecific nonsynonymous polymorphisms is suggestive of balancing selection. It should be mentioned that the MK test is greatly affected by the presence of rare alleles or singleton alleles, and positive values could be produced even for a gene under purifying selection [54,55]. *Pcys230* sequence was used as an outgroup in this test. Fisher's exact test was used to test statistical significance.

3. Results

3.1. Sequence divergence of P230 among Plasmodium species

An amino acid sequence alignment of P230 from *P. vivax* (Pvs230), *P. cynomolgi* (Pcys230), *P. knowlesi* (Pks230), *P. berghei* (Pbs230), *P. chabaudi* (Pchs230), *P. yoelii* (Pys230), *P. falciparum* (Pfs230) and partial sequence of *P. gallinaceum* (Pgs230: aa 1–1527) revealed two distinctive sequence parts: one is an interspecies variable part (IVP) at the N-terminus, in which sequences are highly variable with tandem arrays of oligopeptide repeats, and the other is an interspecies conserved part (ICP), which contains14 cysteine-rich domains (CRDs) in a 7.5 kb region [16,17] (Fig. 1A and Suppl. Fig. 1). The boundary of IVP and ICP does not coincide with the putative cleavage sites proposed for Pfs230 [56], which reside in a C-terminal part of IVP. The cleavage sites are apparently not conserved among other species (Suppl. Fig. 1). In IVP, oligopeptide repeat sequences are species specific: thus, in Pfs230, poly-E and E(E/G)(V/E)G repeats occur as reported earlier [18], poly-(D/E) in Pks230, and degenerative EEEQQ repeat and poly-E in Pcys230 (Fig. 1A and Suppl. Fig. 1). In rodent parasite species, poly-D/E and repeats of EDD are arrayed in Pchs230 and Pbs230, respectively. Pys230 and Pgs230 also contain a D/E-rich sequence region. In Pvs230, there are two repeat sequence regions identified: degenerative repeats of RGXXXGXHXVIH, XVXH, DEDGD and DGND (Fig. 1B). Thus, no primary sequence motifs are shared by *Plasmodium* species examined in the repeat region of P230. It is noteworthy that repeats are rich in acidic residues, E, D and Q. Non-repeat sequences in IVP are also highly divergent, making an alignment not reliable (Suppl. Fig. 1). Despite this, three rodent species show relatively high sequence similarities, and sequences from *P. vivax*, *P. cynomolgi* and *P. knowlesi* show moderate similarities.

ICP is primarily composed of 14 CRDs, in which sequences are somewhat conserved among the seven *Plasmodium* species (Fig. 1A and Suppl. Fig. 1). Each CRD contains 2–6 cysteine residues, all of which are perfectly conserved. In Pvs230, Pcys230 and Pks230, there are two additional cysteine residues identified between CRD10 and CRD11 (Suppl. Fig. 1), which are absent in Pfs230 and P230 of rodent parasites. Amino acid sequence divergence in ICP is 9% to 59% among the seven species, and 21% to 25% between *P. vivax*, *P. cynomolgi* and *P. knowlesi* (Suppl. Table 3A). A phylogenetic tree constructed using aligned sequences (7230 bp) revealed three major monophyletic groups: a group of Pvs230, Pcys230 and Pks230, a group of three rodent P230 s, and Pfs230 (Fig. 2A). When the partial Pgs230 was included in this analysis, Pgs230 was in the same group of Pfs230 (Fig. 2B). The sequence that is most closely related to Pvs230 is Pcys230, and that most distantly related to Pvs230 is Pfs230.

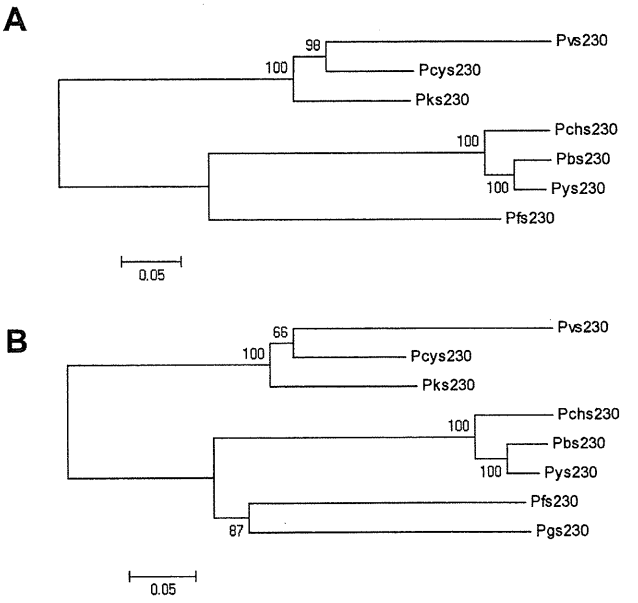

Fig. 2. A phylogenetic tree of *Plasmodium* P230. This tree was constructed by the Neighbor-Joining method. Shown along nodes are bootstrap values with >50%. Nucleotide sequences in ICP among the seven full-length olthologs (A), or among eight olthologs calculated using orthologous sequences to the partial Pgs230 ICP (B).

3.2. Sequence polymorphism in pvs230

We sequenced a total of 112 full-length *pvs230* sequences of isolates collected from Brazil (n = 20), Turkey (n = 20), Madagascar (n = 7), China (n = 20), Thailand (n = 20), Papua New Guinea (n = 21), Solomon Islands (n = 2), Nicaragua (n = 1) and Panama (n = 1) (Suppl. Table 2). The rate of mixed infections, as inferred from superimposed electropherogram peaks, varied greatly, depending on countries, from 0% (Turkey) to 89% (Solomon Islands) (Suppl. Table 2).

In IVP, there are two repeat sequence regions: repeats of 12-mer (RGXXXGXHXVIH) and 4-mer (XVXH) and degenerative repeats of DEDGD and DGND, which are separated by 69 non-repeat amino acids (Fig. 1B). The number of repeats varied among isolates: 2–5 times of 12-mer RGXXXGXHXVIH repeat, and 0–6 times of 4-mer XVXH repeat. In the second repeat, the main repeat motif is DEDGD-VDDD-DGND. Variation in the number of this repeat was limited and geographically restricted: for example, one DEDGD in all countries, except PNG and Solomon Islands, where the repeat number was 0–2 (Suppl. Fig. 2).

In non-repeat sequence regions (8001 bp), there are 77 polymorphic nucleotide sites (Table 1), 40 of which resulting in amino acid replacements (6 sites in IVP and 34 sites in ICP) (Fig. 3). Of the 40 sites, 35 were found in 5 countries (Brazil, Turkey, China, Thailand and PNG), where ≥20 sequences were available in each country for geographical comparisons. Of the 35 sites, 22 sites (60%) were country-specific. A half of these substitutions (12/22) are those with minor allele frequency of ≤5% in the five countries. The number of haplotypes was 72 for worldwide samples with Hd of 0.983 (Table 1). Hd varied geographically, low in Brazil and Turkey and high in Madagascar, China, Thailand, and PNG. In Brazil and Turkey, identical haplotypes were frequently obtained from mul-

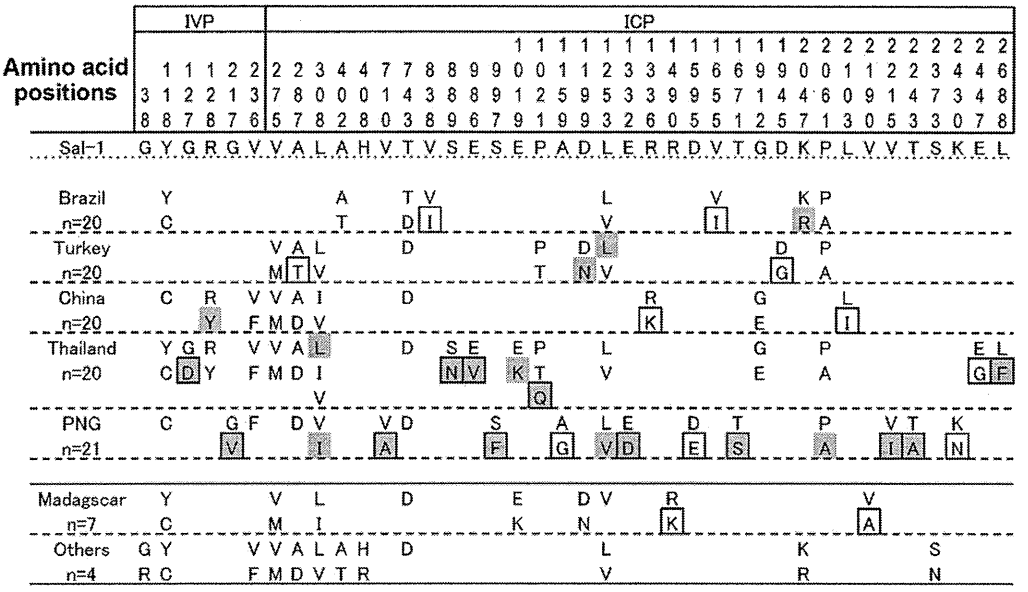

Fig. 3. Amino acid substitutions in Pvs230 of *P. vivax* populations used in this study. IVP and ICP are interspecies variable part and interspecies conserved part, respectively. Amino acid positions are numbered after the Sal-1 sequences. Country-specific amino acid changes are boxed. The category "Others" include Nicaragua, and Panama strains and two isolates from Solomon Islands. Amino acid substitutions with minor allele frequency of ≤0.5% are highlighted in grey.

tiple isolates (Suppl. Fig. 3), contributing to the reduction in Hd. No haplotype was shared by two or more countries, suggesting a strong geographical clustering of *pvs230* alleles.

Nucleotide diversity ($\theta\pi$) was 0.00118 for worldwide samples and 0.00051–0.00084 in six countries (Table 1). $\theta\pi$ was about 1.8-fold higher in non-repeat region of IVP ($\theta\pi = 0.00203$) than in ICP ($\theta\pi = 0.00111$) (Table 1). $\theta\pi$ in each country was lower than that in worldwide samples, suggestive of country-specific substitutions. $\theta\pi$ was relatively high in Thailand and low in Turkey and China. Sliding window plot of $\theta\pi$ revealed a peak ($\theta\pi = 0.01577$) at nucleotide positions 844–943 (positions after the Sal-1 sequence) occurring in CRD1 (Suppl. Fig. 4), in which 5 of a total of 40 amino acid changes in Pvs230 occur. In contrast, in CRD4, where 8 of a total of 27 amino acid changes are clustered in Pfs230 [17], only two amino acid changes occurred in Pvs230. The $\theta\pi$ (0.00118) of *pvs230* in worldwide samples is at least one order lower than that of known blood stage antigen genes such as *pvmsp1*, *pvmsp3a* and *pvdbp* with a few exceptions (Suppl. Table 4).

3.3. Inter-population differentiation of pvs230 among geographic areas

Overall Fst estimate for worldwide populations was 0.51 (95% CI = 0.42–0.56), indicating about half of variation was apportioned within parasite populations (Table 2). This high Fst value suggests a high degree of differentiation of *pvs230* among geographic parasite populations examined. Pairwise comparisons of Fst revealed a significant difference in all pairs, with relatively low values between China and Thailand and high values between PNG and any of other five countries.

3.4. Departure from neutrality

The entire coding sequence (8001 bp), except the N-terminal repeat regions, did show a significant excess of dS over dN in worldwide samples (Table 1). When the sequence was separated into IVP and ICP, a significantly higher dS than dN was noted for ICP (7437 bp) in worldwide samples, suggesting purifying selection. An excess of dS over dN was noted in some (but not in all) countries. In IVP (564 bp), dN was higher than dS in worldwide samples, but this difference was not significant. Tajima's *D* test and Fu and Li's D^* and F^* tests did not detect significant values in all countries and worldwide samples (data not shown).

The MK test for the ICP region detected an excess of fixed synonymous substitutions over nonsynonymous substitutions in worldwide samples ($P = 0.003$) (Suppl. Table 5), again suggesting purifying selection. This excess was not significant in six individual parasite populations, probably due to limited numbers of substitutions. A slightly higher number of intraspecific nonsynonymous substitutions over synonymous substitutions can be seen. However, caution is required for evaluating this difference because the sequences contain a number of singleton mutations (32 singletons in 77 polymorphic sites) (Table 1), suggesting that the balancing selection (positive selection) is unlikely in *pvs230*. The abundance of singleton mutations, most of which are geographic area-specific and thus contributing to high geographic structure (Fst > 0.5 among some areas), limits the validity of the MK test for testing neutrality of *pvs230*.

4. Discussion

The present sequence analysis of P230, a *Plasmodium*-specific gametocyte/gamete surface antigen, identified 2 distinctive parts in the protein: IVP at the N-terminus and ICP, a 7.5-kb component containing 14 CRDs. IVP consists of tandem arrays of species-specific repeats with varying lengths and non-repeat unique sequences, both of which are highly divergent among species, with relatively less divergence in three rodent parasite species and *P. vivax* and related monkey parasites. Despite high divergence, species-specific repeats of P230 are rich in acidic residues, such as D and E. The role of these acidic residue-rich repeats remains unknown. Since the N-terminal repeat region of Pfs230, which induce human antibody and T cell response, is shed into the plasma after processing to form the mature protein during gametogenesis [56], a mechanism for immune evasion by the repeat region has been postulated [57].

In contrast to a highly divergent IVP, ICP is relatively conserved with complete conservation of all cysteine residues in 14

Table 1
Polymorphism in Pvs230 and tests for neutrality.

Area	Region	No. of polymorphic sites	No. of singletons	No. of haplotypes	Haplotype diversity Hd ± SD	Nucleotide diversity $\theta\pi \pm SD$	dN ± SE	dS ± SE	*P* value
Brazil	Entire gene	15	4	6	0.747 ± 0.076	0.00060 ± 0.00007	0.00059 ± 0.00024	0.00065 ± 0.00030	0.8752
(*N* = 20)	IVP	1	0	2	0.395 ± 0.101	0.00070 ± 0.00018	0.00092 ± 0.00092	0.00000 ± 0.00000	0.3105
	ICP	14	4	6	0.747 ± 0.076	0.00059 ± 0.00006	0.00056 ± 0.00027	0.00070 ± 0.00030	0.7405
Turkey	Entire gene	13	2	6	0.763 ± 0.066	0.00054 ± 0.00009	0.00030 ± 0.00011	0.00140 ± 0.00054	0.0496
(*N* = 20)	IVP	0	0	1	0.000 ± 0.000	0.00000 ± 0.00000	0.00000 ± 0.00000	0.00000 ± 0.00000	1.0000
	ICP	13	2	6	0.763 ± 0.066	0.00059 ± 0.00010	0.00032 ± 0.00012	0.00152 ± 0.00062	0.0617
Madagascar	Entire gene	14	9	7	1.000 ± 0.076	0.00063 ± 0.00012	0.00040 ± 0.00016	0.00144 ± 0.00063	0.1171
(*N* = 7)	IVP	1	0	2	0.571 ± 0.119	0.00101 ± 0.00021	0.00133 ± 0.00133	0.00000 ± 0.00000	0.3105
	ICP	13	9	7	1.000 ± 0.076	0.00060 ± 0.00012	0.00033 ± 0.00014	0.00156 ± 0.00067	0.0719
China	Entire gene	13	4	14	0.958 ± 0.028	0.00051 ± 0.00005	0.00050 ± 0.00018	0.00056 ± 0.00035	0.8789
(*N* = 20)	IVP	4	3	4	0.574 ± 0.090	0.00143 ± 0.00038	0.00164 ± 0.00121	0.00075 ± 0.00073	0.5396
	ICP	9	1	14	0.958 ± 0.028	0.00044 ± 0.00003	0.00041 ± 0.00018	0.00054 ± 0.00037	0.7456
Thailand	Entire gene	32	14	18	0.989 ± 0.019	0.00084 ± 0.00010	0.00065 ± 0.00016	0.00153 ± 0.00047	0.0723
(*N* = 20)	IVP	5	1	6	0.726 ± 0.090	0.00230 ± 0.00045	0.00303 ± 0.00172	0.00000 ± 0.00000	0.0728
	ICP	27	13	18	0.989 ± 0.019	0.00073 ± 0.00009	0.00047 ± 0.00015	0.00165 ± 0.00047	0.0241
PNG	Entire gene	28	17	16	0.976 ± 0.020	0.00062 ± 0.00010	0.00030 ± 0.00011	0.00175 ± 0.00056	0.0130
(*N* = 21)	IVP	1	1	2	0.095 ± 0.084	0.00017 ± 0.00015	0.00022 ± 0.00021	0.00000 ± 0.00000	0.2976
	ICP	27	16	16	0.976 ± 0.020	0.00065 ± 0.00011	0.00030 ± 0.00012	0.00189 ± 0.00060	0.0123
Worldwide	Entire gene	77	32	72	0.983 ± 0.005	0.00118 ± 0.00003	0.00089 ± 0.00022	0.00221 ± 0.00064	0.0494
(*N* = 113)	IVP	8	4	9	0.731 ± 0.017	0.00203 ± 0.00013	0.00263 ± 0.00154	0.00013 ± 0.00013	0.1128
	ICP	69	28	71	0.983 ± 0.005	0.00111 ± 0.00003	0.00076 ± 0.00020	0.00238 ± 0.00067	0.0218

Sequence lengths are 564 bp and 7437 by for interspecies variable part and interspecies conserved part, respectively. IVP, interspecies variable part; ICP, interspecies conserved part.

CRDs, which have been predicted to form disulfide bonds in Pfs230 [16,17]. These strongly suggest conformational conservation of the CRDs among all *Plasmodium* species. It remains to be elucidated whether two additional cysteine residues found between CRD10 and CRD11 in P230 from *P. vivax* and related monkey parasites form an additional disulfide bond. Although no strong evidence, suggesting disulfide bonding of the two cysteines, was obtained with available disulfide bond-prediction algorithms (data not shown), a loop structure formed by these two cysteines cannot be excluded. P230 is a member of 6-cys protein family [19], in which Pf12, Pf38 and Pf41 play a role in recognition and invasion of erythrocyte entry by the merozoite [58], and Pbs36 and Pbs36p do so in sporozoite's invasion into liver cells [59]. Pfs230 has been suggested to be associated with male gamete's binding to human erythrocyte during the formation of exflagellation center [20]. Thus, P230 is likely to be involved in interacting with host cells and probably gamete recognition. Such important interactions may impose constraints of sequence variations in Pvs230.

The phylogenetic tree of P230s among eight *Plasmodium* species examined is in good consistency with that of the mitochondrial genomes and 18S rRNA genes [60–62]. In a group of *P. vivax* and *P. vivax*-related monkey malaria species, *P. cynomolgi* P230 is the closest relative to *P. vivax* P230. Genes for gametocytes/gamete surface proteins of rodent malaria parasites are rapidly evolving [19]. Consistent with this, branch lengths of the P230 phylogenetic tree are relatively long, suggesting fast evolution in the P230 gene. A phylogenetic tree of *msp1*, a major immune target merozoite surface protein 1 gene, has recently been shown to significantly differ from that of the mitochondrial genome, with a striking displacement of *P. vivax* from a position close to *P. cynomolgi* in the mitochondrial genome tree to an outlier of Asian monkey parasites, suggesting positive selection in *pvmsp1* [63]. We therefore consider that *pvs230* has not been subjected to such strong positive selection. Positive selection with the ratio of dN/dS over 1 on CRD4 of P230 has recently been detected among three rodent species [19]. In *P. vivax*, *P. cynomolgi* and *P. knowlesi*, dN/dS over 1 was not detected (data not shown), suggesting that positive selection in the P230 gene is lineage specific.

The observed polymorphism level of *pvs230* ($\theta\pi = 0.00118$) is much lower than that of most blood stage antigen genes (Suppl. Table 4). Since blood stage antigens are targets of host antibody responses, it is likely that high genetic diversity in these antigens is a mechanism for parasite's immune evasion. Balancing selection has been inferred to maintain high levels of polymorphism in *pvmsp1* [43,63], *pvmsp3a* [64], *pvmsp3β* [65], *pvmsp5* [66,67], and *pvdbp* [68]. In *pvs230*, however, no evidence for balancing selection was obtained in this study but data suggest purifying selection in this gene. Functional and/or structural constrains of Pvs230 would probably limit the accumulation of point mutations, resulting in low level of polymorphism. A higher nucleotide diversity in IVP than in ICP also supports this notion because in Pfs230, most parts of IVP is presumed to be cleaved off from the mature protein containing CRDs. Selective sweep, as often seen in drug resistant genes, potentially contributes to reduction in polymorphism. In this case, only variant haplotypes become predominant in a population. Selective sweep is unlikely for *pvs230* because haplotype diversity was high in most parasite populations. Recent population growth may also reduce genetic diversity; however, significantly negative values were not obtained by Tajima's *D* test and Fu and Li's *D** and *F** tests, making population expansion unlikely for *pvs230*. A comparison of polymorphism between *pvs230* and *pfs230*, although the number of *pfs230* sequences analyzed was small [17], reveals a difference in the distribution of polymorphism across the genes: major amino acid substitutions is clustered within CRD4 in Pfs230, whereas it is within CRD1 in Pvs230. Different intragenic distribution of polymorphisms between *P. falciparum* and *P. vivax* may

Table 2
Inter-population differentiation (Fst) of pvs230 between six geographic areas.

	Brazil	Turkey	Madagascar	China	Thailand	PNG
Brazil		<0.00001	<0.00001	<0.00001	<0.00001	<0.00001
Turkey	0.548		<0.00001	<0.00001	<0.00001	<0.00001
Madagascar	0.337	0.416		<0.00001	<0.00001	<0.00001
China	0.484	0.559	0.508		<0.00001	<0.00001
Thailand	0.453	0.457	0.366	0.219		<0.00001
PNG	0.604	0.673	0.611	0.526	0.425	

Overall Fst is 0.505 (95% CI, 0.423–0.564) with variance of 5.104. Fst values are shown in the bottom left and *P* values are shown in the upper right.

suggest different constraints in these distantly related *Plasmodium* species.

This study showed a high genetic differentiation (Fst = 0.51) of *pvs230* among global parasite populations. This indicates very limited gene flow between populations. Some proteins on the surface of gametes are involved in gamete recognition and fertilization, and genes for these proteins tend to be highly divergent among populations [69,70]. This has been suggested to be true for Pfs48/45, in which Fst is as high as 0.69 among parasite populations from Africa, Asia and South America [71], given that Fst of two housekeeping genes of *P. falciparum* populations is 0.20 for worldwide parasite populations [41]. Fst of *pfs230* is not known. Previous estimates of Fst among geographic *P. vivax* populations, which have been derived from microsatellite data (0.13–0.26 between Southeast Asia and Colombia [72]) and predominantly silent single-nucleotide polymorphisms (0.228 between Brazil and Asia [73]), are remarkably lower than the Fst of *pvs230* obtained in this study. Comparisons of Fst between *P. vivax* housekeeping genes and *pvs230* as well as other gamete surface protein genes, such as *pvs47* and *pvs48/45* would be required to assess divergent (directional) selection on *P. vivax* gamete surface protein genes.

In conclusion, the limited polymorphism of *pvs230* observed in this study would provide a strong ground for developing effective TBV based on Pvs230, and help to identify polypeptide regions suitable for designing vaccines. Since it would not be practical to include all 14 CRDs in a TBV, several CRDs that can effectively induce transmission blocking immunity (TBI) should be targeted. The present study identified short sequence regions with relatively high polymorphism, particularly in the N-terminal part of CRD1. In our parallel studies, we have found that a recombinant DNA vaccine, encompassing CRD1 of Pvs230, induces TBI (Tachibana et al., unpublished). Whether polymorphism in small polymorphic regions in CRD1 is involved in strain-specific TBI awaits further studies. Additionally, this study revealed a substantial endemic area-specific SNPs in *pvs230*. If a vaccine includes a region having such polymorphism, caution is required, and the reactivity of the serum antibodies to variants and hence efficiency of TBI in individuals living in endemic areas should be monitored.

Disclosure

The view of the author, J. Sattabongkot, does not purport to reflect the position of the US Department of the Army or Department of Defense.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.028.

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N-Terminal Prodomain of Pfs230 Synthesized Using a Cell-Free System Is Sufficient To Induce Complement-Dependent Malaria Transmission-Blocking Activity▽

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The aim of a malaria transmission-blocking vaccine is to block the development of malaria parasites in the mosquito and thus prevent subsequent infection of the human host. Previous studies have demonstrated that the gametocyte/gamete surface protein Pfs230 can induce transmission-blocking immunity and have evaluated *Escherichia coli*-produced Pfs230 as a transmission-blocking vaccine candidate. In this study, we used the wheat germ cell-free expression system to produce N-terminal fragments of Pfs230 and evaluated the transmission-blocking activity of antisera raised against the recombinant Pfs230 protein. The rabbit antisera reacted to the surface of cultured gametocytes and gametes of the *Plasmodium falciparum* NF54 line, recognized the 360-kDa form of parasite-produced Pfs230 by Western blot assay, and reduced the infectivity of NF54 parasites to *Anopheles stefensi* mosquitoes in the presence of complement in a standard membrane feeding assay. Thus, our data demonstrate that the N-terminal pro domain of Pfs230 is sufficient to induce complement-dependent transmission-blocking activity against *P. falciparum*.

Malaria is a major infectious disease caused by protozoa of the genus *Plasmodium* and is transmitted by anopheline mosquitoes. There were an estimated 225 million clinical cases of malaria and 781,000 malaria-related deaths in 2009 (39). Among four species of human malaria parasites, *Plasmodium falciparum* causes the most severe form of malaria and is globally distributed. Elimination strategies are, however, limited and confounded by the emergence of multidrug-resistant parasites and insecticide-resistant mosquitoes (15). Therefore, the development of malaria vaccines is an essential component of malaria elimination and, further, eradication (13, 14). Malaria vaccines are generally divided into three groups based on the parasite life cycle target stage: preerythrocytic, asexual blood stage, and transmission-blocking vaccines (TBVs). Preerythrocytic vaccines act against sporozoites and liver stage parasites and are intended to prevent infection. Asexual blood stage vaccines are aimed at reducing parasite multiplication and growth to protect against clinical symptoms. TBVs block malaria transmission by interrupting the parasite life cycle in the mosquito. Thus, TBVs are considered to be essential components of combination vaccines that target multiple stages of the parasite's life cycle aimed at malaria eradication (1, 30).

Target antigens for TBV development are sexual and mosquito stage-specific surface molecules (5). Antigens expressed on the surface of zygotes and ookinetes in the mosquito midgut (e.g., P25 and P28 in *P. falciparum* and *P. vivax*), referred to as postfertilization antigens, have been shown to be effective for inducing transmission-blocking immunity (8, 17, 20, 31). Ookinete surface antigens, Pfs25 in *P. falciparum* and Pvs25 in *P. vivax*, have been tested in phase I clinical trials, and a positive correlation of TBV efficacy with antibody titers has been demonstrated (18, 22, 44). However, P25 is not expressed by blood stage parasites and hence it is not exposed to the human immune response (7, 27); therefore, the anti-Pfs25 immune response will not be boosted by natural malaria infection. In contrast to ookinete surface antigens, antigens that are expressed on male and female gametes, such as Pfs48/45 and Pfs230 (prefertilization antigens), may be boostable by natural malaria infection, as they are also expressed on gametocytes during infection and are thus exposed to the human immune system (7, 27). The major proteins found on the surface of both male and female gametocytes/gametes belong to a family of six cysteine-containing domain proteins designated cysteine motif (CM) domains that include Pfs48/45 (21) and Pfs230 (41). Pfs230 is a 360-kDa protein that contains 7 CM domains (Fig.

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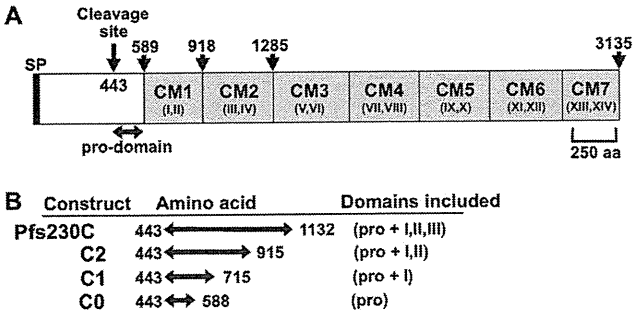


FIG. 1. Pfs230 primary structure and design of constructs. (A) Schematic representation of predicted structural motifs for Pfs230. SP represents a signal peptide. The cleavage site at amino acid (aa) 443 represents the site at which processing of Pfs230 occurs during gamete formation. The region comprising amino acids 443 to 588 refers to the pro domain. CM1 through CM7 (regions shaded in gray) represent CM domains as described by Williamson et al. (43). Amino acid positions (arrows) 589, 918, 1285, and 3135 represent the starts of CM1, CM2, and CM3 and the end of CM7, respectively. Also, domains I through XIV, as described by Gerloff et al. (11), are in parentheses. (B) Construct Pfs230C spans the pro domain through domain III and comprises amino acids 443 to 1132. Construct Pfs230C2 spans the pro domain through domain II and comprises amino acids 443 to 915. Construct Pfs230C1 spans the pro domain and domain I and comprises amino acids 443 to 715. Construct Pfs230C0 spans the pro domain only and comprises amino acids 443 to 588.

1A) (6, 11, 36) and elicits a humoral immune response in infected individuals that can mediate transmission-blocking immunity (12, 16). Consequently, Pfs230 as a component of a vaccine offers the advantage that it will elicit an immune response that is boosted by natural infection and will thus provide long-lasting immunity (3, 28).

The monoclonal antibodies (MAbs) recognizing the CM domain of Pfs230 potently block the infectivity of the *P. falciparum* parasites to mosquitoes (26, 28, 29, 37). Several regions of Pfs230 have been expressed as maltose-binding protein (MBP) fusions in *Escherichia coli* (designated r230/MBP) (43). Antibodies raised against an N-terminal 76-kDa fragment of Pfs230 designated r230/MBP.C (amino acids 443 to 1132) were bound to the surfaces of gametes and reduced the infectivity of *P. falciparum* to mosquitoes in the presence of complement (43). This was the first demonstration of the transmission-blocking activity of antibodies against a recombinant Pfs230 antigen. However, r230/MBP.C protein elicited incomplete oocyst reduction. Attempts to increase the efficacy of Pfs230C-based vaccines have included yeast expression systems (38), DNA vaccine (9), and vaccinia virus expression systems (40). Disappointingly, these attempts to produce a more efficacious vaccine have failed, with r230/MBP.C appearing to be the most potent Pfs230 TBV identified to date (40). Recently, we reported that among the various protein synthesis systems, the wheat germ cell-free protein synthesis system is permissive for the production of correctly folded malaria proteins (30, 32). The wheat germ cell-free system was demonstrated to produce malaria proteins without any codon optimization (33). With the expectation that the wheat germ cell-free system would produce higher-quality Pfs230 proteins more effective in eliciting transmission-blocking activity than the *E. coli*-expressed r230/MBP.C protein, we decided to express recombinant Pfs230C and its truncated forms and to characterize the minimal region that contains the immunodominant epitope(s) of Pfs230C sufficient to induce malaria transmission-blocking activity.

MATERIALS AND METHODS

Cloning of fragments encoding truncated forms of Pfs230. Williamson et al. (43) predicted the CM domains, CM1 through CM7, in Pfs230 (PFB0405w) (Fig. 1A). Gerloff et al. (11) also described cysteine-rich domains I through XIV in Pfs230. Based on those predictions, we synthesized four different truncated forms of Pfs230, i.e., Pfs230C (pro domain through domain III, amino acids 443 to 1132 with 13 cysteines), Pfs230C2 (pro domain through domain II, amino acids 443 to 915 with 8 cysteines), Pfs230C1 (pro domain and domain I, amino acids 443 to 715 with 4 cysteines), and Pfs230C0 (pro domain, amino acids 443 to 588 without any cysteines) (Fig. 1B). Genomic DNA encoding each of the truncated forms of Pfs230C was amplified by PCR from *P. falciparum* 3D7 DNA and cloned between the XhoI and NotI sites of plasmid pEU-E01-GST-TEV (a vector with an N-terminal glutathione *S*-transferase tag followed by a tobacco etch virus protease cleavage site; CellFree Sciences, Matsuyama, Japan). The inserted nucleotide sequences were confirmed using the ABI PRISM 3130 Genetic Analyzer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Production and purification of truncated Pfs230 proteins. Recombinant proteins Pfs230C, Pfs230C2, Pfs230C1, and Pfs230C0 were produced with the wheat germ cell-free protein expression system by the bilayer translation reaction method described previously (33, 34). After their synthesis, the truncated forms of Pfs230C were affinity purified by passage through a glutathione-Sepharose 4B column (GE Healthcare, Camarillo, CA) and eluted by on-column cleavage with AcTEV protease (Invitrogen, Carlsbad, CA) after extensive washing of the column with phosphate-buffered saline (PBS). Concentrations of purified proteins were determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and the bands were visualized with Coomassie brilliant blue (Fig. 2). Purified protein samples were stored in aliquots at −80°C until further use.

Preparation of rabbit antisera. A Japanese White rabbit for each antigen was immunized subcutaneously 3 times at 3-week intervals with 250 μg of each purified truncated Pfs230 protein emulsified with complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) for the prime and with incomplete Freund's adjuvant (Sigma-Aldrich) for the first and the second boosts. Blood was collected before immunization and 2 weeks after the third immunization. Antisera were prepared as previously described (25). Preimmune sera from individual rabbits were used as controls.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Ehime University, and the experiments were conducted according to the Ethical Guidelines for Animal Experiments of Ehime University.

Enzyme-linked immunosorbent assay (ELISA). The titers of the IgG in the rabbit antisera raised against different truncated Pfs230 proteins were measured by ELISA. Briefly, 96-well MaxiSorp ELISA plates (Nunc, Rochester, NY) were coated with 0.5 μg/ml recombinant truncated forms of Pfs230 in 20 mM borate buffer (pH 8.9) and incubated overnight at 4°C. After blocking with 2 mg/ml gelatin in 20 mM borate buffer, serum samples were plated in duplicate at a starting dilution of 1:100 with PBS containing 0.01% Tween 20 and 2 mg/ml gelatin and titrated in 10-fold dilutions. Target-specific IgG was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at 1:2,000 (Biosource, Camarillo, CA) and visualized using 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS; Wako, Osaka, Japan) as the substrate. The reaction was then stopped with 0.1 M citric acid, and the optical density values were measured. Reciprocal serum dilutions that gave a mean absorbance value of 0.5 at 415 nm were determined as the endpoint titers. In order to measure the antibody titers against native Pfs230 protein, stage V gametocyte of the *P. falciparum* NF54 line was extracted with PBS with 1% Triton X-100 containing Complete Proteinase Inhibitor Cocktail (Roche, Indianapolis, IN) and centrifuged at 21,900 × *g* for 20 min. The collected supernatant was diluted to a final concentration of 1.0 μg protein/ml and used as a capture antigen, and ELISA was performed as described above.

Immunofluorescence assay (IFA). An indirect IFA was performed with cultured gametocytes or macrogametes/zygotes after the induction of gametogenesis from the cultured gametocytes of the *P. falciparum* NF54 line (43). Air-dried thin smears of the parasites were prepared on glass slides and stored at −80°C

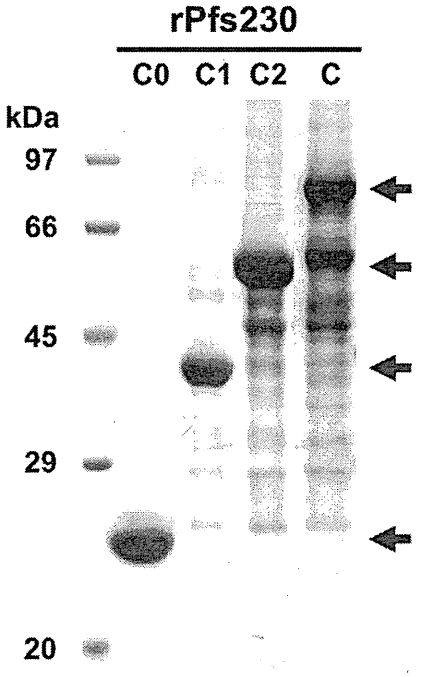


FIG. 2. SDS-PAGE analysis of the purified truncated recombinant Pfs230 (rPfs230) proteins expressed. The different truncated Pfs230 proteins, Pfs230C0 (C0), Pfs230C1 (C1), Pfs230C2 (C2), and Pfs230C (C), were expressed in the wheat germ cell-free system and separated on an SDS-12.5% polyacrylamide gel under reducing condition and stained with Coomassie brilliant blue (arrows indicate the expected truncated Pfs230 proteins). The extra bands other than those indicated by arrows in lanes C2 and C are translation products due to premature termination of translation.

until use. The smears were thawed, fixed with ice-cold acetone for 3 min, and blocked with PBS containing 5% nonfat milk (PBS milk) at 37°C for 30 min. They were then incubated with either rabbit immune serum (1:500 dilution) or matched preimmune serum as a negative control at 37°C for 1 h, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) as a secondary antibody (1:500) at 37°C for 30 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/ml) mixed with a secondary antibody solution. Slides were mounted in ProLong Gold Antifade reagent (Invitrogen) and observed under a 63× oil immersion lens. High-resolution image capture and processing were performed using a confocal scanning laser microscope (LSM5 PASCAL; Carl Zeiss MicroImaging, Thornwood, NY). Images were processed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Western blot analysis. Proteins of the cultured gametocytes were extracted in nonreducing SDS-PAGE loading buffer and boiled at 98°C for 3 min, and extract from approximately 10^5 gametocytes per lane was subjected to electrophoresis on a 12.5% polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were then transferred to a 0.2-μm polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The proteins were immunostained with either preimmune or immune serum as the primary antibody. To ensure that equal amounts of the protein samples were loaded in each lane for Western blot analysis, the membranes were simultaneously probed with anti-*Pf*HSP70 mouse MAb (4C9) as a quantitative marker for parasite proteins (35). The membranes were then probed by HRP-conjugated goat anti-rabbit IgG antibody (GE Healthcare) together with HRP-conjugated goat anti-mouse IgG antibody (GE Healthcare) and visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on a LAS 4000 mini luminescent image analyzer (GE Healthcare). The relative molecular masses of the proteins were estimated with reference to Precision Plus Protein Standards (Bio-Rad, Hercules, CA).

Transmission-blocking assays. The transmission-blocking activity of the sera was tested by an *ex vivo* standard membrane feeding assay (SMFA) as described previously (44). Briefly, an *in vitro* gametocyte culture of *P. falciparum* (NF54 line) was evaluated for the percentage of stage V gametocytes (>0.5%), and the vitality of exflagellation centers was observed at ×400 magnification. The gametocyte pellet was diluted with normal O^+ red blood cells (Interstate Blood Bank, Memphis, TN) and a normal heat-inactivated (complement minus) or unheated (complement plus) AB^+ human serum pool (Interstate Blood Bank, Memphis, TN) to achieve a 0.15% ± 0.05% concentration of stage V gametocytes and a hematocrit of 50%. This infected blood mixture was kept at 37°C and aliquoted into 200-μl portions prior to feeding. One 200-μl aliquot of "infected blood" was mixed with 60 μl of the test serum diluted with a normal heat-inactivated O^+ human serum pool at 1:1, 1:2, or 1:4 for final dilution of the sera in feeders at 1:5.3, 1:11, and 1:21, respectively. The mixture was immediately fed to 3- to 8-day-old *Anopheles stephensi* (Nijmegen strain) mosquitoes prestarved for 24 to 30 h through a membrane feeding apparatus using a thin stretched Parafilm membrane. Mosquitoes were kept for 7 to 8 days after feeding at 26°C under high-humidity conditions to allow parasites to develop into oocysts. Infectivity was measured by dissecting at least 20 mosquitoes per sample, staining the midguts with a 0.05% merbromin (Mercurochrome) solution in water for at least 20 min, and counting the oocysts in each midgut. The percent reduction of the oocyst count per mosquito was determined by the formula $100 \times$ (mean oocyst $no._{negative\ control}$ − mean oocyst $no._{test}$)/mean oocyst $no._{negative\ control}$, where the negative-control feeding used preimmune serum from the same rabbit. All samples or diluted samples were tested in replicate. To verify whether the difference in oocyst numbers between preimmune and immune groups is statistically significant, data were analyzed by comparing the medians of two groups using the Mann-Whitney U test. Oocyst prevalence was statistically analyzed for all vaccination regimens by Fisher's exact test. Probability (P) values of less than 0.05 were considered statistically significant in both analyses.

RESULTS

Synthesis of soluble Pfs230C proteins in a wheat germ cell-free system. In order to evaluate the transmission-blocking activities elicited by the wheat germ cell-free expressed truncated forms of Pfs230C and to define the minimal region of Pfs230 sufficient to induce the malaria transmission-blocking activity, we designed different Pfs230C constructs, i.e., Pfs230C (pro domain through domain III, amino acids 443 to 1132 with 13 cysteines), Pfs230C2 (pro domain through domain II, amino acids 443 to 915 with 8 cysteines), Pfs230C1 (pro domain and domain I, amino acids 443 to 715 with 4 cysteines), and Pfs230C0 (pro domain, amino acids 443 to 588 without any cysteines), based on schematic diagrams of Pfs230 (Fig. 1A and B) without codon optimization and expressed them in a wheat germ cell-free system (Fig. 2). Figure 2 shows the different truncated Pfs230 proteins resolved in a 12.5% SDS-polyacrylamide gel. Almost all of the truncated Pfs230 proteins were recovered in the supernatant fraction and easily purified as a single dominant band (Fig. 2, arrows) along with other, nonspecific, faint bands by affinity chromatography in lanes C2 and C. The yields of purified Pfs230C0, Pfs230C1, Pfs230C2, and Pfs230C proteins were 62, 54, 65, and 75 μg/5.0 ml of the reaction mixture, respectively. These results demonstrate that the wheat germ cell-free system is able to translate the native Pfs230 gene sequences and produce soluble proteins.

Rabbit antibodies against truncated forms of Pfs230C recognized native parasite proteins. To evaluate the immunogenicity of the recombinant truncated forms of Pfs230, rabbit antisera were collected on study days 0 (pre) and 56 (post). ELISA analysis of the IgG responses elicited by the four truncated forms of Pfs230C (Fig. 3A) showed that all of the postimmune sera contained high titers of antibodies, with no discernible difference among the four recombinant protein formulations, i.e., titers against all of the recombinant proteins, Pfs230C0 ($10^{5.8}$), Pfs230C1 ($10^{6.1}$), Pfs230C2 ($10^{5.8}$), and Pfs230C ($10^{6.0}$) (Fig. 3A).

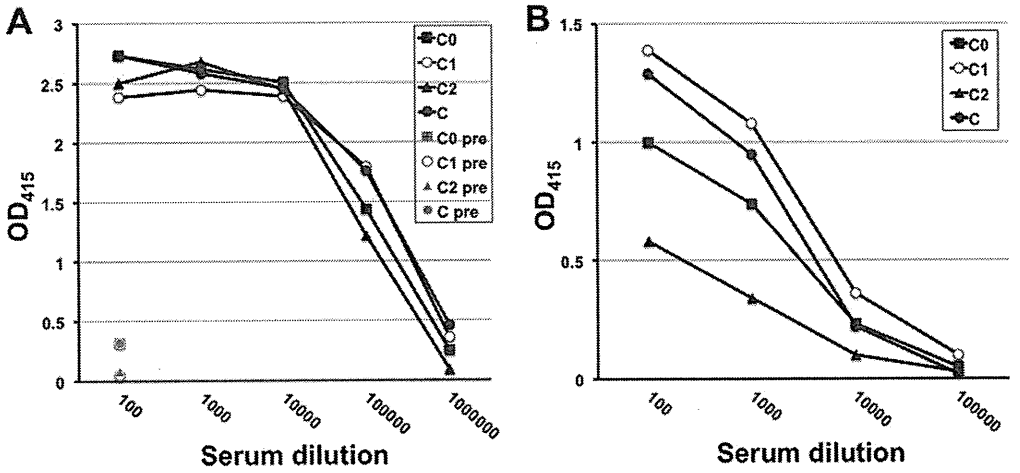


FIG. 3. IgG responses elicited by immunization with different truncated forms of Pfs230 in rabbits. (A). Serum IgG titers in samples collected before antigen administration (pre) and on day 56 postimmunization using recombinant Pfs230C0, -C1, -C2, and -C as ELISA capture antigens. (B). Serum IgG titers in samples collected on day 56 postimmunization using native, parasite-derived Pfs230 as the plate antigen. Reciprocal serum dilutions that gave a mean absorbance at 415 nm of 0.5 were determined as the endpoint titers. OD, optical density.

All of the negative-control preimmune sera showed no IgG responses (Fig. 3A).

To evaluate the immunoreactivity of the antisera against parasite-derived native proteins, Western blot analysis was performed. Extract from stage V gametocytes of the *P. falciparum* NF54 line was separated by 12.5% SDS-PAGE, and specific bands with the expected mobility of native Pfs230 protein (Fig. 4, arrow) were detected under nonreducing conditions (Fig. 4, left panel) using antiserum against each recombinant Pfs230 protein. Preimmune serum failed to recognize the native Pfs230 protein (Fig. 4, right panel). These results suggest that the recombinant truncated forms of Pfs230C prepared by the cell-free system as soluble proteins retained native epitopes. To ensure that the same amount of each gametocyte protein sample was loaded in each lane for Western blot analysis, the membranes were also probed with anti-PfHSP70 MAb (4C9) (35). The intensities of the PfHSP70 bands indicated that the amounts of samples loaded in the lanes were comparable (Fig. 4, arrowhead). Importantly, ELISA analysis of the IgG responses elicited by the four truncated forms of Pfs230C showed that the postimmune serum against each recombinant protein, Pfs230C0 (titer, $10^{3.5}$), Pfs230C1 (titer, $10^{3.8}$), Pfs230C (titer, $10^{3.7}$), or Pfs230C2 (titer, $10^{2.4}$) contained significant levels of antibodies with no discernible difference in the levels of antibodies against the parasites' native Pfs230 protein extracted from the gametocytes of *P. falciparum* (Fig. 3B).

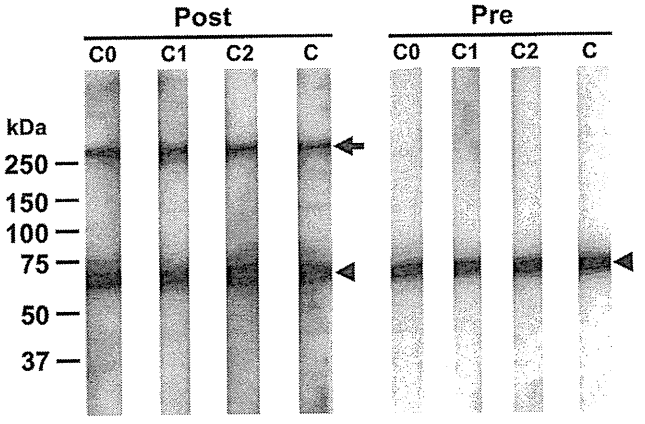


FIG. 4. Western blot analysis using antisera against different truncated forms of Pfs230, Pfs230C0 (C0), Pfs230C1 (C1), Pfs230C2 (C2), and Pfs230C (C). Extracts prepared from stage V gametocytes of the *P. falciparum* NF54 line were separated on SDS-12.5% polyacrylamide gels under nonreducing condition and transferred onto PVDF membrane. Proteins on PVDF membranes were immunostained with either rabbit anti-Pfs230C0, -C1, -C2, and -C sera (lanes C0, C1, C2, and C in the left panel) or the corresponding preimmune (Pre) sera (right panel) (arrow). The relative molecular masses of the proteins were estimated with reference to Precision Plus Protein Standards (Bio-Rad, Hercules, CA). To ensure that equal amounts of the protein samples were loaded into the lanes for Western blot analysis, the membranes were simultaneously probed with anti-*Pf*HSP70 mouse MAb (4C9) as a quantitative marker of parasite protein (35) (arrowhead).

To confirm the specificity of the antisera for the truncated forms of Pfs230, IFA was performed against stage V gametocytes and gametes of *P. falciparum* (Fig. 5). Antisera against the recombinant truncated forms of Pfs230 specifically stained the surfaces of both gametocytes and gametes (Fig. 5). All of the negative-control preimmune sera showed no staining of the parasites (data not shown).

Antibodies against truncated forms of Pfs230 reduce parasite transmission to the mosquito vector in the presence of complement. The transmission-blocking activity of the rabbit antisera raised against recombinant Pfs230C was evaluated by SMFA. The antisera were diluted to minimize nonspecific effects of sera on transmission-blocking activity, and the preimmune sera from individual animals were used as negative controls. The multiple assays were performed on different days using the cultured *P. falciparum* NF54 line (Table 1). The number of oocysts formed in the presence of complement in experiment 1 was significantly lower ($P < 0.0001$), by 86%, than that obtained with the preimmune serum (with complement, experiment 1). A similar result (88% reduction) was obtained ($P < 0.0001$) in experiment 2 (with complement,

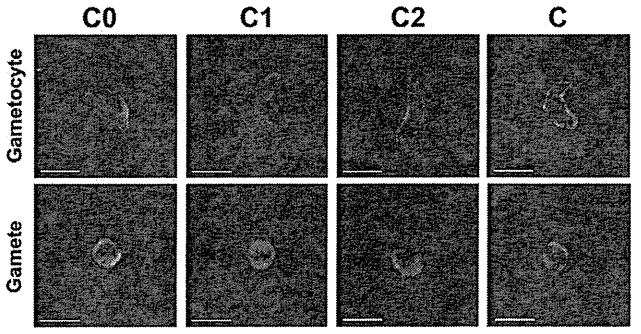


FIG. 5. Reactivity of antisera against Pfs230C0, -C1, and -C2 and Pfs230C in immunofluorescence microscopy. Samples prepared from stage V gametocytes (upper panels) and gametes (lower panels) of the *P. falciparum* NF54 line were immunostained with the antisera indicated above the panels. Immunostained images were visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (green). Nuclei were stained with DAPI (blue). Scale bars, 5 μm.

experiment 2). In the third experiment (Table 1), a further reduction was observed ($P < 0.0001$, 99.0%) when a 1:5.3 dilution of anti-Pfs230C antiserum was tested in the presence of complement. The number of oocysts was inversely proportional to the concentration of antiserum added (experiment 3 in Table 1), and the differences between pre- and postimmune groups, even at the lowest concentration of the antiserum (1:21) tested were still significant ($P < 0.0001$).

To identify the minimal functional transmission-blocking domains within Pfs230C, the complement-dependent transmission-blocking activity was also evaluated in sera raised against Pfs230C0, -C1, and -C2 (Fig. 1B). As shown in Table 1, the antisera raised against Pfs230C0, -C1, and -C2 (with complement, experiment 4) significantly reduced the number of oocysts per mosquito if complement was present in the SMFA. Surprisingly, the rabbit antisera (other than anti-Pfs230C0) reduced oocyst formation significantly in the mosquito, even in the absence of complement (without complement, experiment 4). Consistent with observations in SMFAs in general, the apparent transmission-blocking efficacy was lower when the oocyst load of the preimmune control was higher (Table 1, experiment 4), and it was higher when the oocyst load of the preimmune control was lower (Table 1, experiment 5).

The oocyst prevalence, expressed as the number of oocyst-positive mosquitoes over the total number of mosquitoes dissected (numbers infected/dissected in Table 1), was statistically analyzed for all vaccination regimens by Fisher's exact test. Both the number of oocyst and the oocyst prevalence in mosquitoes were significantly reduced by the Pfs230C (1:5.3) antiserum in experiment 3 and the Pfs230C0 (1:5.3), Pfs230C1 (1:5.3), and Pfs230C2 (1:5.3) antisera in experiment 5.

DISCUSSION

We have demonstrated that the wheat germ cell-free system supported the production of soluble recombinant Pfs230C (comprising amino acids 443 to 1132 of Pfs230), which induced transmission-blocking antibodies (Table 1). In previous studies (4, 43), transmission-blocking antibodies against Pfs230C were effective only at a higher concentration (i.e., a 1:2 dilution). However, in this study, the transmission-blocking antibodies induced by Pfs230C were effective even at a lower concentration (i.e., a 1:5.3 dilution). The specific IgG titers of these immune sera were comparable when they were tested against the immunizing antigens; the IgG titers of the anti-Pfs230C2 serum was slightly lower than those of the other sera when they were tested against native, parasite-derived Pfs230. The transmission-blocking efficacy is complement dependent, as previously reported (40). Moreover, even when the antiserum was further diluted (1:21 dilution in Table 1, experiment 3), a 70% reduction of the oocyst number was still achieved ($P < 0.0001$). These results suggest that the transmission-blocking efficacy seen is comparable to that of *E. coli*-produced recombinant Pfs230C, i.e., r230/MBP.C comprising amino acids 443 to 1132.

Evidence from previous studies and the data presented herein that recombinant Pfs230C has transmission-blocking activity, defining a minimal region of Pfs230 sufficient to induce the malaria transmission-blocking activity, will facilitate the development of candidate Pfs230 TBV into a vaccine. Bustamante et al. (4) expressed the regions of recombinant truncated Pfs230 proteins in *E. coli* as MBP fusions, i.e., r230/MBP.C (amino acids 443 to 1132), r230/MBP.C5′ (amino acids 443 to 791), r230/MBP.CM1 (amino acids 583 to 913), r230/MBP.C1 · 6 (amino acids 453 to 913), and r230/MBP.C2 (amino acids 914 to 1268), and used them to generate antibodies in mice. All of the antisera recognized native Pfs230 on the surfaces of the gametes, but only antibodies against r230/MBP.C, and not antibodies against other truncated forms, reduced the number of oocysts. The authors therefore concluded that the entire Pfs230C protein is required to produce the transmission-blocking epitope (4). However, our results demonstrate that antibodies against all of the recombinant truncated forms of Pfs230 reduced the number of oocysts not only in the presence but even in the absence of active complement (Table 1). The reason for the transmission-blocking activity in the absence of active complement may be due to the presence of antibodies that block biological function such as blocking of the fertilization of gametes besides the antibodies that are involved in the complement-mediated lysis of gametes in polyclonal antibodies. These results demonstrate that the topology of the native Pfs230 domains may be better retained in the recombinant truncated forms of Pfs230 synthesized in the eukaryotic wheat germ cell-free system than in the proteins expressed in *E. coli*. Importantly, there is no obvious difference in the transmission-blocking efficacy of antibodies raised against Pfs230C0, -C1, or -C2. This suggests that the transmission-blocking epitope is confined to the N-terminal cysteine-free pro domain (Pfs230C0). Therefore, the entire Pfs230C protein is not required for immunization to induce complement-dependent transmission-blocking activity against *P. falciparum*, but the minimal N-terminal cysteine-free pro domain (Pfs230C0) is sufficient (Table 1, experiments 4 and 5).

Several TBV candidates have complex structures and multiple disulfide bonds, which hamper their production, evaluation, and development as recombinant vaccine candidates (18, 19). However, the N-terminal pro domain of Pfs230 (Pfs230C0) is cysteine free and hence not constrained by disulfide bonds. The absence of disulfide bonds is expected to facilitate the production and development of Pfs230C0 as a TBV candidate. Future vaccine development efforts should

TABLE 1. Evaluation of sera from rabbits immunized with Pfs230Cs by SMFA[a]

Antigen and expt no. (dilution)	Sample[b]	With complement				Without complement			
		No. inf./diss.[c]	Median (IQR[e])	% Reduction[f]	P[g]	No. inf./diss.	Median (IQR)	% Reduction	P
Pfs230C									
1 (1:5.3)	Rabbit 2 pre	24/25	14.0 (8.5–33.0)			22/24	12.5 (5.0–41.5)		
1 (1:5.3)	Rabbit 2 post	18/23	2.0 (1.0–4.0)	86	<0.0001	23/25	11.0 (5.5–26.0)	34	NS[h]
Pfs230C									
2 (1:5.3)	Rabbit 2 pre	23/23	21.0 (11.0–28.0)			23/24	17.0 (8.0–20.0)		
2 (1:5.3)	Rabbit 2 post	21/23	2.0 (1.0–3.5)	88	<0.0001	23/25	8.0 (5.5–15.5)	33	NS
Pfs230C									
3 (1:5.3)	Rabbit 2 pre	21/22	57.0 (30.0–76.0)						
3 (1:5.3)	Rabbit 2 post	6/23[d]	0.0 (0.0–1.0)	99	<0.0001				
3 (1:11)	Rabbit 2 pre	22/22	62.0 (29.5–88.0)						
3 (1:11)	Rabbit 2 post	20/21	3.0 (1.0–6.5)	94	<0.0001				
3 (1:21)	Rabbit 2 pre	22/22	55.0 (37.0–73.0)						
3 (1:21)	Rabbit 2 post	23/24	16.5 (12.0–24.0)	70	<0.0001				
Pfs230C0									
4 (1:5.3)	Rabbit 100 pre	21/21	111.0 (78.5–134.5)			22/22	58.0 (39.5–105.0)		
4 (1:5.3)	Rabbit 100 post	20/23	20.0 (13.0–28.0)	82	<0.0001	20/21	53.0 (33.0–82.5)	8	NS
Pfs230C1									
4 (1:5.3)	Rabbit 101 pre	21/21	81.0 (66.0–120.0)			20/21	84.0 (68.0–101.0)		
4 (1:5.3)	Rabbit 101 post	22/22	30.5 (14.5–45.5)	63	<0.0001	24/24	53.5 (16.5–83.5)	38	<0.01
Pfs230C2									
4 (1:5.3)	Rabbit 102 pre	21/21	120.0 (80.0–166.5)			21/21	67.0 (39.5–90.0)		
4 (1:5.3)	Rabbit 102 post	22/22	33.5 (20.5–52.5)	71	<0.0001	26/26	38.0 (16.0–63.5)	46	<0.05
Pfs230C0									
5 (1:5.3)	Rabbit 100 pre	18/22	2.0 (1.0–5.0)			23/23	15.0 (10.0–21.0)		
5 (1:5.3)	Rabbit 100 post	6/28[d]	0.0 (0.0–0.0)	92	<0.0001	24/24	5.0 (3.0–7.5)	62	<0.0001
Pfs230C1									
5 (1:5.3)	Rabbit 101 pre	22/24	6.0 (3.0–8.0)			22/24	14.0 (8.5–16.0)		
5 (1:5.3)	Rabbit 101 post	8/23[d]	0.0 (0.0–1.0)	92	<0.0001	22/24	6.5 (2.5–10.0)	41	<0.05
Pfs230C2									
5 (1:5.3)	Rabbit 102 pre	18/24	2.5 (0.5–4.0)			28/28	9.0 (5.5–15.5)		
5 (1:5.3)	Rabbit 102 post	8/23[d]	0.0 (0.0–1.0)	79	<0.001	25/25	6.0 (3.5–8.0)	48	<0.01

[a] One 200-μl aliquot of "infected blood" with a hematocrit of 50% was mixed with 60 μl of the test serum diluted with a normal heat-inactivated O^+ human serum pool in a 1:1, 1:2, or 1:4 final dilution of the sera in feeders at 1:5.3, 1:11, and 1:21, respectively.

[b] The serum sample from an individual rabbit was used. Numbers such as 2, 100, 101, and 102 are animal identification numbers.

[c] Oocyst prevalence, expressed as the number of oocyst-infected mosquitoes over the total number of mosquitoes dissected (inf./diss.), was statistically analyzed between pre- and postimmune serum groups for all vaccination regimens by Fisher's exact test.

[d] Statistically significant, $P < 0.05$.

[e] IQR, interquartile range.

[f] Percent reduction in the oocyst count per mosquito was determined by the formula $100 \times (\text{mean oocyst no.}_{\text{negative control}} - \text{mean oocyst no.}_{\text{test}})/\text{mean oocyst no.}_{\text{negative control}}$, where the negative-control feeding used preimmune serum from the same rabbit.

[g] The median number of oocysts was statistically analyzed by comparing the day 56 immune serum with the matched preimmune rabbit serum (Mann-Whitney U test), and P values were obtained.

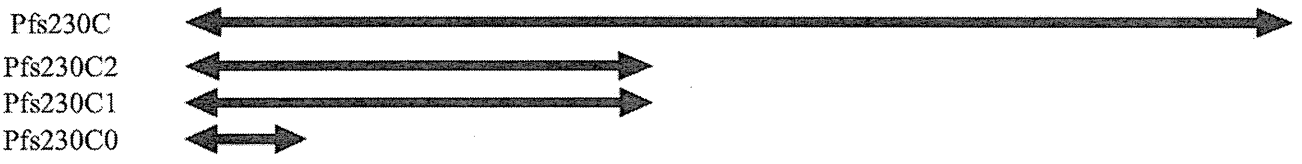
[h] NS, not significant.

focus on vaccine formulation or delivery methods to increase the immunogenicity of Pfs230C0.

It is critical to design a vaccine based on sequences with minimum polymorphisms to avoid parasite evasion of vaccine-induced immunity. The native Pfs230 protein is expressed on the surface of gametocytes, and field studies have found that antibodies against Pfs230 are generated during natural infection (12, 16). Exposure to host immune pressure has resulted in Pfs230 sequence polymorphisms observed in field isolates (11, 24, 42). A compilation of single nucleotide polymorphisms (SNPs) in Pfs230 sequences from 13 laboratory strains deposited in PlasmoDB (http://plasmodb.org/plasmo/) showed that Pfs230C contains a total of 10 nonsynonymous SNPs. Pfs230C2 and Pfs230C1 contain 4 of them, and, importantly, Pfs230C0 does not contain any nonsynonymous SNPs (Table 2). These data further support the notion that Pfs230C0 is a promising TBV candidate.

Production of functionally active truncated forms of Pfs230 will also facilitate understanding of the dynamics of naturally acquired transmission-blocking immunity and assist with the future evaluation and deployment of TBVs in populations living in areas where malaria is endemic. Recently Bousema et al.

TABLE 2. Nonsynonymous SNP sites of Pfs230 deposited in PlasmoDB[a]



Strain	443–588[b]	605[b]	661[b]	701[b]	713[b]	963[b]	1008[b]	1043[b]	1069[b]	1082[b]	1087[b]
3D7	—[c]	G	K	V	D	T	N	Q	V	I	S
K1	—	S	—	—	—	I	—	—	—	M	—
Ghana 1	—	S	N	—	—	—	—	—	—	—	—
D10	—	S	—	—	—	I	—	K	—	M	—
HB3	—	S	—	—	—	—	—	—	—	—	Y
7G8	—	S	—	—	—	—	—	—	—	—	—
Dd2	—	S	—	—	—	I	—	K	—	M	—
Senegal 3404	—	S	N	—	—	—	H	—	—	—	—
RO33	—	—	—	—	—	—	H	—	—	M	—
D6	—	—	—	—	Y	—	—	—	—	—	—
Santa Lucia	—	—	—	—	—	—	—	—	I	M	—
V1_S	—	S	—	V	—	—	—	K	—	—	—
FCB	—	—	—	—	—	—	—	—	—	—	—

[a] The data were obtained from the PlasmoDB version 7.1 (http://plasmodb.org/plasma).
[b] Amino acid position(s) in the Pfs230 amino acid sequence based on the 3D7 sequence used as a reference.
[c] —, same amino acid residue as in the 3D7 sequence.

(3) reported that the antibody response to Pfs230 is boosted by exposure to gametocytes. To date, most of those studies used the sandwich ELISA method using anti-Pfs230 MAbs in combination with cultured *P. falciparum* gametocyte crude lysates (2). In the case of TBV application in future field trials, seroepidemiological surveillance of such naturally existing transmission-reducing immunity seems to be essential; however, the availability of crude parasite antigen for such a large-scale study is one of the hurdles to be overcome. Our study has proven that we could synthesize properly folded soluble truncated forms of Pfs230 in large quantities using the wheat germ cell-free system. Therefore, the availability of antigens for such a large-scale seroepidemiological surveillance will no longer be limiting.

The present study demonstrates the potential of wheat germ cell-free protein synthesis for the production of Pfs230C for clinical studies. However, to date, there is no cGMP facility using the cell-free system for the production of recombinant proteins for clinical studies. Therefore, expression trials based on the plant-based transient protein expression system applicable to the downstream cGMP (23) are under way toward the development of Pfs230-based TBV (10).

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A Plant-Produced Pfs230 Vaccine Candidate Blocks Transmission of *Plasmodium falciparum*▽†

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***Plasmodium falciparum* is transmitted to a new host after completing its sexual cycle within a mosquito. Developing vaccines against the parasite sexual stages is a critical component in the fight against malaria. We are targeting multiple proteins of *P. falciparum* which are found only on the surfaces of the sexual forms of the parasite and where antibodies against these proteins have been shown to block the progression of the parasite's life cycle in the mosquito and thus block transmission to the next human host. We have successfully produced a region of the Pfs230 antigen in our plant-based transient-expression system and evaluated this vaccine candidate in an animal model. This plant-produced protein, 230CMB, is expressed at approximately 800 mg/kg in fresh whole leaf tissue and is 100% soluble. Administration of 230CMB with >90% purity induces strong immune responses in rabbits with high titers of transmission-blocking antibodies, resulting in a greater than 99% reduction in oocyst counts in the presence of complement, as determined by a standard membrane feeding assay. Our data provide a clear perspective on the clinical development of a Pfs230-based transmission-blocking malaria vaccine.**

Malaria is a severe, and at times fatal, mosquito-borne disease caused by a protozoan parasite. The most severe form of the disease is caused by *Plasmodium falciparum*. Hundreds of millions of malaria cases occur around the world each year, with close to one million deaths (http://www.who.int/mediacentre/factsheets/fs094/en/index.html). The development of vaccines against malaria is a critical component in the control of the infection and elimination of the disease, as parasites and mosquitoes are growing resistant to the current methods of chemical intervention (10, 14). The focus is on developing vaccines targeting different stages of the parasite's life cycle, including transmission-blocking (TB) vaccines (TBVs) that can inhibit the sexual stages of parasite development in the mosquito midgut, thus alleviating the transmission pressure and the disease burden at the population level (3, 45). After a blood meal on infected humans, mosquitoes become infected by ingesting a sexual form of the malaria parasite called gametocytes (reviewed in reference 1). Subsequent sporogenic development in the mosquito can be prevented by the presence of antimalarial TB antibodies in the ingested blood meal. Thus, TBVs are directed against the parasite's sexual stages in the mosquito and are designed to halt the development of oocysts and the subsequent production of infective sporozoites (4). It has been proposed that TBVs should be considered a major part of a malaria eradication program aimed at combining vaccines against multiple stages of the parasite's life cycle to maximize efficacy (reviewed in reference 20).

Development of TBVs has been explored for over 20 years, with the candidate targets falling into two classes: (i) antigens that are present on the surface of *P. falciparum* gametocytes and gametes (such as Pfs230 and Pfs48/45) and are expressed in the human host, where immunity against them could potentially be boosted by natural infection, and (ii) antigens that are expressed on the zygote and ookinete stages (such as Pfs25 and Pfs28) and are not expressed in the human host (32, 43, 47). In the 1980s, incidents of serum-mediated TB activity were reported in individuals infected with *Plasmodium vivax* (25) and serum samples collected during field studies contained antibodies against the gametocyte surface proteins Pfs230 and Pfs48/45 (12, 15, 17, 27, 29, 33, 36). Additionally, Graves et al. (15) and Healer et al. (17) showed a positive correlation between the abilities of sera to immunoprecipitate ^{125}I-labeled Pfs230 and to block *P. falciparum* transmission in a standard membrane feeding assay (SMFA). Antibodies in these sera recognize Pfs230 only under nonreducing conditions, suggesting that recognition is conformation dependent, and further studies revealed that Pfs230-associated TB activity is complement dependent (18, 34, 35, 37). Thus, Pfs230 has been targeted as a potential candidate for TBV development. Further-

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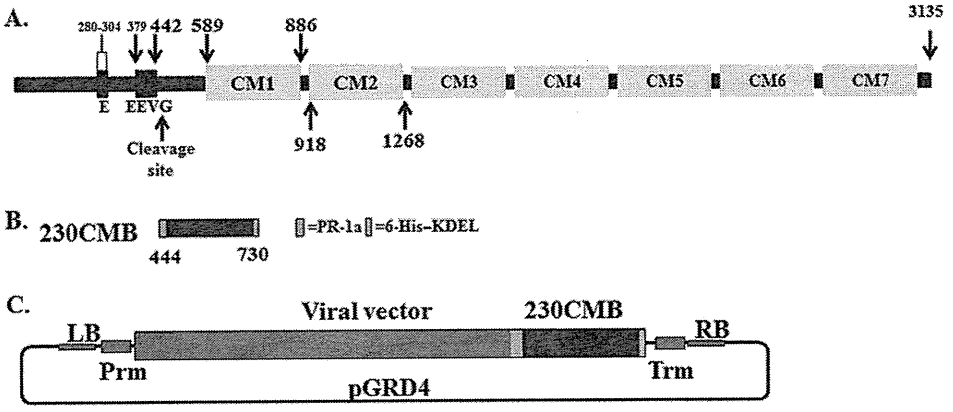

FIG. 1. Pfs230 genetic organization and construct design. (A) Schematic representation of predicted structural motif for Pfs230, including the approximate amino acid range of each relevant region or domain. The cleaved portion of Pfs230 contains the 25 glutamic acid repeat region, E, and the 16 tandem EEVG repeat region and precedes the seven paired cysteine motif domains, CM1 to CM7. (B) 230CMB includes aa 444 to 730 and contains the PR-1a signal peptide at the N terminus and a 6-His tag and the KDEL ER retention signal at the C terminus (light gray boxes). (C) Diagram of the launch vector for target expression containing the *Agrobacterium* left border (LB) and right border (RB) sequences, the plant promoter (Prm) and terminator (Trm) regulatory elements, the viral vector launch sequences, and the Pfs230CMB target sequence.

more, the immune response to a Pfs230 TBV may be boosted by natural infection, potentially providing long-lasting immunity, and may be advantageous to a Pfs230-containing vaccine (34, 47, 48).

Pfs230 is a 363-kDa protein that contains 70 cysteine residues. Carter et al. (5) predicted that Pfs230 has seven paired domains and that the TB target epitopes are located within these motif-defined domains. Preceding the conserved paired domains are a region of 25 glutamic acids (amino acids [aa] 280 to 304) and a region of 16 tandem repeats of the tetramer EEVG (aa 379 to 442). Both of these regions are processed from the N-terminal end of the protein upon release of the gametocyte from the erythrocyte, and antibodies generated against these glutamate-rich regions do not bind to gametes (2). Gerloff et al. (13) defined repeated structures of cysteine motifs from aa 589 to aa 3135 and predicted the complex disulfide bonding necessary for correct folding and conformation. Recombinant expression of full-length Pfs230, due to its complexity (13), has not been accomplished, but different regions have been expressed as fusions to the maltose-binding protein (MBP) in *Escherichia coli* (48). Antibodies against one such region of Pfs230, designated C (aa 443 to 1132), are able to immunoprecipitate radiolabeled Pfs230, bind to the surface of gametes, and, in the presence of complement, reduce the infectivity of *P. falciparum* to mosquitoes. This was the first report of induction of TB antibodies against a recombinant Pfs230 antigen that is immunogenic during natural malaria infection and the first step in TBV development (48). However, this r230/MBP.C construct elicits only partial oocyst reduction. The MBP fusion was produced in the cytosol of *E. coli* in the absence of disulfide bond formation; thus, it is possible that only a small fraction of the recombinant protein correctly displays the native epitopes, therefore resulting in low levels of TB activity.

In an effort to dissect functional regions within the sequences corresponding to r230/MBP.C, Tachibana et al. (40a) produced a recombinant Pfs230 domain C (aa 443 to 1132) and its truncated forms Pfs230C0 (aa 443 to 588), Pfs230C1 (aa 443 to 715), and Pfs230C2 (aa 443 to 915) in a wheat germ cell-free expression system (41). Rabbit antibodies raised against these recombinant proteins displayed significant TB activities in the SMFA (40a). However, the wheat germ cell-free expression system presents a challenge for large-scale manufacturing of recombinant proteins for clinical applications, and to date there is no facility compliant with current good manufacturing practices (cGMP) guidelines. The plant-based transient protein expression platform developed by the Fraunhofer Center for Molecular Biotechnology (CMB) allows for manufacturing of recombinant vaccine antigens under cGMP, and the ongoing clinical testing of two vaccine candidates (H1N1 and H5N1 influenza viruses) produced at CMB's pilot plant facility supports the feasibility of this system.

In our study, we have engineered and produced a portion of Pfs230 domain C, 230CMB, corresponding to aa 444 to 730 of Pfs230, using our plant-based expression system (28). The protein was purified and characterized by biophysical methods, and its immunogenicity and TB activity were assessed in rabbits. Results showed that serum antibodies generated against 230CMB were able to bind to the parasite with high efficiency in immunofluorescence assays (IFAs) and showed a greater than 99% reduction in oocyst counts in the presence of complement. These data indicate that the 230CMB antigen has great potential as a TBV candidate.

MATERIALS AND METHODS

Cloning and expression analysis. After the region comprising aa 444 to 730 of Pfs230 was optimized for plant expression (GeneArt, Regensburg, Germany), it was cloned into the launch expression vector pGRD4 (Fig. 1C) (28, 38) for expression in plants. The target construct included sequences encoding the signal peptide of plant pathogenesis-related protein 1a (PR-1a) (24) for entry into the secretory pathway, the KDEL sequence for retention in the endoplasmic reticulum (ER), and a 6-histidine (6-His) tag for ease of purification and detection. The expression constructs were then electroporated into *Agrobacterium tumefaciens* strain GV3101, and the resulting bacteria were grown overnight in minimal medium (16). The optical density (OD) of the cultures was determined, and the protein expression strain was mixed with an *Agrobacterium* strain expressing a suppressor of silencing, p19 protein (46). The *Agrobacterium* solution was introduced by manual infiltration into the leaves of 6-week-old, soil-grown *Nicotiana*

benthamiana plants as described previously (16). Plant leaf tissue samples were collected 3 to 7 days postinfiltration to determine the levels of expression and solubility of the target protein. Samples were weighed and extracted in three volumes of a phosphate-based buffer containing 0.5% Triton X-100 for extraction of total soluble protein or with the addition of gel loading buffer (50 mM Tris HCl [pH 6.8], 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol, 100 mM dithiothreitol) for extraction of total protein. Proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Target protein expression levels were assessed by Western blot analysis using an anti-His mouse monoclonal antibody (MAb; Roche, Indianapolis, IN) or a target-specific MAb (LMIV; NIH) as the primary antibody and a horseradish peroxidase (HRP)-labeled anti-mouse antibody as the secondary antibody (Jackson ImmunoResearch, West Grove, PA). Results were analyzed using the GeneGnome system (Syngene, Frederick, MD). Peak days of expression and solubility profiles were determined. Constructs of interest were vacuum infiltrated on a larger scale into hydroponically grown *N. benthamiana* for protein purification (16).

Purification. Aerial plant tissue (500 g) was harvested, homogenized in extraction buffer containing 20% glycerol, and incubated for 20 min at 4°C in the presence of 0.5% Triton X-100. Following incubation, the plant homogenate was clarified by centrifugation (15,000 × *g* for 45 min). The supernatant was then filtered through Miracloth (Calbiochem, Gibbstown, NJ) and passed through a 10-in. BioLife filtration capsule (Cuno; 3M, Meriden, CT) and a 0.2-μm Sartopore filter cartridge (Sartorius, Bohemia, NY). The filtrate was loaded onto an XK 26 column with Chelating Sepharose Big Beads resin (70-ml column volume [CV]) using the ÄKTA Purifier 100 (GE Healthcare, Piscataway, NJ) at a flow rate of 25 ml/min. The column was washed with 5 CVs of 50 mM sodium phosphate (pH 7.5)–0.5 M NaCl–20 mM imidazole–0.5% Triton X-100 and then with 10 CVs of 50 mM sodium phosphate (pH 7.5)–0.5 M NaCl–20 mM imidazole. The target was eluted with 50 mM sodium phosphate (pH 7.5)–0.5 M NaCl–20% glycerol–300 mM imidazole at a flow rate of 3.5 ml/min. Immobilized metal affinity chromatography (IMAC) eluant was dialyzed into 10 mM sodium phosphate (pH 7.0)–10% glycerol, and the resulting material was centrifuged at 78,000 × *g* for 10 min to remove the potential insoluble material. The clarified and dialyzed sample was loaded at a flow rate of 3 ml/min onto three 5-ml HiTrap Capto Q ion-exchange chromatography columns attached in tandem, and the target was eluted with 300 mM NaCl at a flow rate of 3 ml/min. The purified target was dialyzed into saline (0.9% NaCl) and filtered through a 25-mm 0.2-μm nylon syringe filter (Fisher Scientific, Pittsburgh, PA) prior to freezing and storage at −70°C.

Protein characterization. SDS-PAGE was performed using 10% acrylamide gels stained with Coomassie (Gel Code Blue, Pierce, Rockford, IL) as described previously (21). When Western blot assays were performed (40), samples were transferred to PVDF membranes and blocked with I-Block (Applied Biosystems, Carlsbad, CA), and the target protein was detected using anti-6-His (Qiagen, Valencia, CA or Roche, Indianapolis, IN) or anti-Pfs230 MAb (2G5 or 2B10; NIH) as the primary antibody and HRP-labeled anti-mouse (Jackson ImmunoResearch, West Grove, PA) antibody as the secondary antibody. N-terminal sequencing was performed by Edman degradation (Proteos, Inc., Kalamazoo, MI). Recognition of plant-produced 230CMB by Pfs230-specific TB MAbs was determined by enzyme-linked immunosorbent assay (ELISA) using 96-well MaxiSorp plates (Nunc, Rochester, NY) coated with 1 μg/ml 230CMB in phosphate-buffered saline (PBS) and incubated overnight at 4°C. After blocking with 0.5% I-Block in PBS with 0.1% Tween 20, MAbs 2G5 and 2B10 were plated in triplicate at starting concentrations of 5 μg/ml and 2 μg/ml, respectively, and titrated in 5-fold dilutions. The MAbs were detected using HRP-conjugated goat anti-mouse IgG at 1:7,500 (Jackson ImmunoResearch, West Grove, PA) and visualized using *o*-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, St. Louis, MO) as a substrate with an acid stop. Size exclusion chromatography (SEC)-multiangle laser light scattering (MALLS) experiments were performed using a Superdex 200 column (10 by 300 mm; 13-μm particle size) with inline UV (GE) and MALLS (Wyatt) detectors to determine the molecular weight of the purified target. The purified 230CMB protein was analyzed at a 1-mg/ml concentration (100 μg) and injected with 50% sample loop overfill. The dilution (where needed) and running buffer was PBS (pH 7.5). The SEC-MALLS flow rate was 0.5 ml/min at room temperature. Notably, when determining the molar extinction coefficient (ε), we use the method of Edelhoch (8). The sum of the specific contributions leading toward total absorption at 280 nm for each residue (Trp, Tyr, and Cys) is determined based on the nominal amino acid sequence.

Rabbit immunogenicity study. Plant-produced 230CMB protein was evaluated for immunogenicity and TB potential in rabbits. Groups of 5 rabbits were immunized with 100 μg of 230CMB on study days 0 and 28 either subcutaneously emulsified in complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) for the prime followed by incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO) for the boost or intramuscularly adsorbed to Alhydrogel (Brenntag-Biosector, Frederikssund, Denmark). Control animals were immunized with PBS plus adjuvant. Serum samples from immunized rabbits were collected on study days 0, 28, 42, and 56 and assessed for total IgG titers by ELISA. The presence of gametocyte-binding antibodies in sera from study day 42 was determined using an IFA on fixed gametocytes. If results from the IFA were positive, the samples were then tested in a suspension IFA (SIFA) using live macrogametes/zygotes. Following positive SIFA results, the day 42 serum samples were then tested for the presence of TB antibodies in an SMFA. All animal protocols were approved by the Institutional Animal Care and Use Committee at Covance Research Products, Inc. (Denver, PA).

ELISA. 230CMB-specific serum IgG titers were measured by ELISA using 96-well MaxiSorp plates (Nunc, Rochester, NY) coated with 1 μg/ml 230CMB in PBS and incubated overnight at 4°C. After blocking with 0.5% I-Block in PBS with 0.1% Tween 20, serum samples were plated in duplicate at a starting dilution of 1:100 and titrated in 5-fold dilutions. Target-specific IgGs were detected using HRP-conjugated goat anti-rabbit IgG at 1:7,500 (Jackson ImmunoResearch, West Grove, PA) and visualized using OPD (Sigma-Aldrich, St. Louis, MO) as a substrate with an acid stop. Reciprocal serum dilutions that gave a mean absorbance value four times greater than the background were determined as the endpoint titers.

IFA. An indirect IFA was performed with a mixture of cultured sexual and asexual stage *P. falciparum* (isolate NF54) parasites air dried onto a multispot slide as previously described (30). Briefly, parasites were incubated with a 1:100 dilution of the test sera in PBS, rinsed with PBS, and incubated with Alexa Fluor 488-labeled chicken anti-rabbit IgG (H+L; Invitrogen). The slides were rinsed, washed, mounted under a coverslip, and examined under UV illumination with a Leitz Ortholux fluorescence microscope (×500 magnification).

SIFA. As described previously for SIFA analysis (30, 37), gametocytes were allowed to undergo gametogenesis. Subsequently, a suspension of 10^6 macrogametes/zygotes (100 μl) was mixed with 100 μl of a 1:100 dilution of the sera in PBS and incubated for 20 min on ice. Parasites were washed with 1 ml of PBS, collected by centrifugation for 3 min at 3,000 rpm (benchtop centrifuge), and incubated with 50 μl of Alexa Fluor 488-labeled chicken anti-rabbit IgG (H+L; Invitrogen). After a wash with PBS, the cells were examined as described for the IFA.

SMFA. Antisera obtained from rabbits immunized with the 230CMB antigen were assessed for TB activity by SMFA as previously described (22, 31, 37). Briefly, 90 μl of rabbit serum was mixed with 30 μl of naïve human serum (as a source of complement) and 150 μl of *in vitro* gametocyte culture of *P. falciparum* (NF54 line). The mixture was fed to *Anopheles stephensi* mosquitoes through a membrane feeding apparatus. Preimmune sera, sera from rabbits immunized with PBS alone, and MAb 63F2A2 (37) served as the controls. All serum samples were tested separately at a final dilution of 1:3 in the feeder assay in the presence of active or heat-inactivated (56°C, 30 min) human complement. Complement was collected from type AB blood from Dutch blood bank donors with no previous malaria exposure as described previously (37). Fully engorged mosquitoes were separated and held at 26°C. Seven days later, midguts of 20 mosquitoes were examined for oocysts. The observed TB activity of serum was determined as the percentage reduction in the arithmetic mean oocyst number in test samples compared to that in paired controls (42). The experiment was considered valid when at least 70% of the mosquitoes feeding on control sera were infected. For comparison of groups and to assess for a statistically significant difference between the groups, data were analyzed by a nonparametric test (not normally distributed) by comparing the medians of two groups using the Mann-Whitney test or by comparing three or more groups using the Kruskal-Wallis test followed by post tests. If significance was indicated, Dunn's analysis was used for comparison with the control group.

RESULTS

230CMB is expressed in *N. benthamiana* as a soluble protein. Schematic diagrams of the Pfs230 antigen and the expression vector are shown in Fig. 1A and B. Region C of Pfs230 (aa 443 to 1132) starts in the EEVG repeat region and continues to the middle of the second cysteine motif domain of the protein. We have engineered the N-terminal portion of region C spanning aa 444 to 730, which we designate 230CMB (Fig. 1C), into our launch vector system (28, 38) and transformed

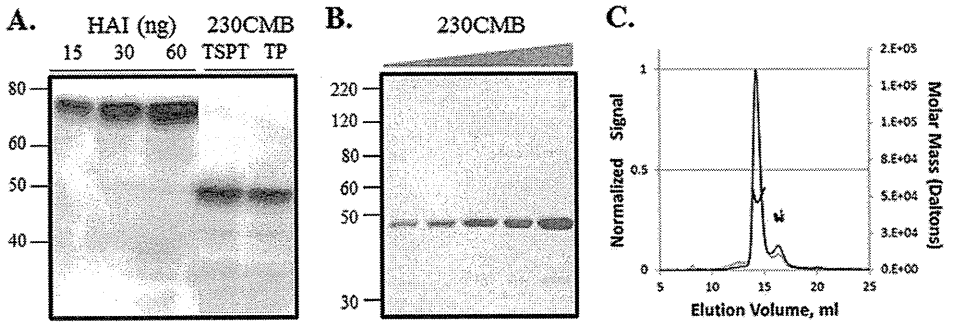

FIG. 2. Expression analysis and *in vitro* characterization of the purified 230CMB antigen produced for immunological evaluation. (A) Crude plant extract consisting of total soluble protein with 0.5% Triton X-100 (TSPT) and total protein (TP) were resolved by SDS-PAGE, transferred to PVDF membrane, detected with anti-4-His MAb, and compared to different amounts of the standard (plant-produced hemagglutinin from the A/Indonesia/05/05 strain of influenza virus [HAI]). (B) Increasing amounts of purified 230CMB were resolved by SDS-PAGE and stained with Gel Code Blue. Size comparisons to MagicMark XP (A) or BenchMark (B) molecular size markers were made. The values to the left of panels A and B are molecular sizes in kilodaltons. (C) SEC of 230CMB followed by UV and light scattering detection. The solid black trace is UV280, the thin dotted trace is the light scatter signal (Rayleigh ratio), and the markers indicate the calculated molar mass over the given elution volume.

the resulting plasmid into *A. tumefaciens* GV3101 for expression in plants. The target gene sequence, prior to cloning, was optimized for plant expression by GeneArt and included sequences encoding the PR-1a signal peptide (24) at the N terminus and the ER retention sequence (KDEL) and a 6-His tag, to aid in purification, at the C terminus. Following infiltration of *N. benthamiana* plants with the *Agrobacterium* strain harboring 230CMB, expression (in the range of 800 mg/kg of fresh leaf tissue) and solubility (100%) of 230CMB in leaf tissue were confirmed (Fig. 2A). Other constructs designed for expression of different Pfs230 region C fragments (aa 444 to 1132, 444 to 886, and 585 to 700) have also been tested but were down-selected due to a low target expression level or poor target solubility.

Purification and biophysical characterization of 230CMB produced in *N. benthamiana*. Vacuum infiltration of hydroponically grown *N. benthamiana* was performed for purification of 230CMB for further studies. Protein was purified as described in Materials and Methods. Following IMAC, 230CMB was approximately 75% pure, a value that was improved to 95% purity, as determined by Coomassie staining of an SDS-PAGE gel, after anion-exchange chromatography. A single predominant band was visible on a Coomassie-stained SDS-PAGE gel (Fig. 2B) and was also detected using an anti-6-His tag-specific MAb in a Western blot assay (data not shown). The nominal molecular mass of 230CMB is 34 kDa; however, it is resolved at approximately 48 kDa under reducing denaturing conditions. The greater molecular mass of 230CMB determined under reducing denaturing conditions can potentially be explained by the glycosylated nature of the product. Indeed, upon treatment with peptide *N*-glycosidase F, 230CMB has been shown to migrate at a slightly lower molecular weight (unpublished data). This, however, does not exclude the possibility of other posttranslational modifications or O-linked glycosylation leading to the larger-than-expected-size molecule.

The 230CMB preparation was further characterized to determine the molecular weight and solution state profile of the product by SEC-MALLS. SEC of 230CMB, followed by UV and light scattering detection, indicated one primary peak with a molar mass distribution of 48 kDa, while the small signal at the 8-ml column void volume indicates a very small amount of a high-molecular-weight species in the protein sample (Fig. 2C).

To confirm correct processing of the signal peptide, the protein was subjected to N-terminal sequencing performed by Edman degradation and was shown to have the expected N terminus (YVDEK). In addition, plant-produced 230CMB reacted with two anti-Pfs230 MAbs, 2B10 and 2G5, which were shown to be TB antibodies. 230CMB was detected by these two MAbs with endpoint titers, determined at a level 4-fold over the background, of greater than 50,000 for each MAb, indicating that the TB epitopes in our plant-produced protein are recognized by these antibodies.

230CMB elicits TB antibodies in rabbits. To evaluate the immunogenicity and TB activity of 230CMB, rabbits were immunized with 100 μg of 230CMB with Freund's adjuvant (subcutaneously) or with Alhydrogel (intramuscularly) using a prime-boost regimen on days 0 and 28. Serum samples were collected on study days 0, 28, 42, and 56. ELISA analysis of the IgG responses elicited by the two 230CMB vaccine formulations showed that protein emulsified in Freund's adjuvant generated a 9-fold higher IgG titer than that adsorbed onto Alhydrogel. However, the kinetics of the response was the same for both formulations, with antibody titers peaking on study day 42 and remaining elevated through day 56 (Fig. 3). Importantly, day 42 sera from animals immunized with both formulations contained a high titer of antibodies that bound to parasites in both IFA and SIFA. Sera from the Freund's adjuvant-treated group of animals had a higher serum IFA titer (≥12,800) than sera from the group of animals receiving antigen adsorbed on Alhydrogel (= 800), and both serum samples were positive in the SIFA at a dilution of 1:250.

Further evaluation of sera in the SMFA in the presence of complement demonstrated that all animals immunized with 230CMB emulsified in Freund's adjuvant or adsorbed on Alhydrogel generated antibodies that resulted in ≥99% TB (Table 1). As was anticipated, in the absence of complement, the levels of mean oocyst reduction were less pronounced, with the group receiving antigen emulsified in Freund's adjuvant ex-