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Identification of domains within Pfs230 that elicit transmission blocking antibody responses

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Abstract

A transmission-blocking vaccine (TBV) against *Plasmodium falciparum* is likely to be a valuable tool in a malaria eradication program. Pfs230 is one of the major TBV candidates, and multiple Pfs230-based vaccines induced antibodies, which prevented oocyst formation in mosquitoes as determined by a standard membrane-feeding assay (SMFA). Pfs230 is a >300 kDa protein consisting of 14 cysteine motif (CM) domains, and the size and cysteine-rich nature of the molecule have hampered its production as an intact protein. Except for one early study with maltose-binding protein fusion Pfs230 constructs expressed in *Escherichia coli,* all other studies have focused on only the first four CM domains in the Pfs230 molecule. To identify all possible TBV candidate domains, we systematically produced either single-CM-domain (a total of 14), 2-CM-domain (7), or 4-CM-domain (6) recombinant protein fragments using a eukaryotic wheat germ cell-free expression system (WGCFS). In addition, two more constructs which covered previously published regions, and an N-terminal prodomain construct spanning the natural cleavage site of Pfs230 were produced. Antisera against each fragment were generated in mice and we evaluated the reactivity to native Pfs230 protein by Western blots and immunofluorescence assay (IFA), and functionality by SMFA. All 30 WGCFS-produced Pfs230 constructs were immunogenic in mice. Approximately half of the mouse antibodies specifically recognized native

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Conflicts of interests

The authors declare that they have no competing interests.

Appendix A. Supplementary material

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Pfs230 by Western blots with variable band intensities. Among them, seven antibodies showed higher reactivities against native Pfs230 determined by IFA. Interestingly, antibodies against all protein fragments containing CM domain 1 displayed strong inhibitions in SMFA, while antibodies generated using constructs without CM domain 1 showed no inhibition. The results strongly support the concept that future Pfs230-based vaccine development should focus on the Pfs230 CM domain 1.

Keywords

Malaria; Pfs230; *Plasmodium falciparum*; Transmission-blocking vaccine; Wheat germ cell-free system

1. Introduction

Morbidity and mortality of malaria have been reduced significantly in the last 15–20 years, and the World Health Organization (WHO) identified more than 20 countries that had the potential to eliminate malaria by the year 2020 [1]. However, there were still 445,000 estimated malaria-related deaths in 2016, mostly due to *Plasmodium falciparum,* and the spread of resistance against existing drugs and insecticides has been a serious concern [1]. Vaccine development against *P. falciparum* malaria has targeted all stages of its complicated life cycle, but one of the advantages of a transmission-blocking vaccine (TBV) is that the transmission stage is the biological bottleneck [2]; the majority of wild-caught mosquitoes or mosquitoes which directly fed from malaria-infected volunteers showed fewer than 5–6 oocysts (one of the mosquito-stage parasites) per mosquito. Therefore, a TBV that can prevent infection of mosquitoes following feeding on an infectious blood meal has the potential to accelerate elimination and eventual eradication of malaria-causing parasites [2,3]. TBVs are designed to induce antibodies in human hosts against sexualstage malaria antigens or to antigens expressed in the mosquito vector, and these antibodies can inhibit parasite development in the mosquito when they are ingested with parasites.

Pfs230 is one of the major TBV candidates and plays an important role in sexual-stage development of the parasite. The full length Pfs230 expressed in gametocyte (sexual-stage parasites in humans) is a 360-kDa protein. When a gametocyte is ingested by a mosquito, the parasite egresses from the erythrocyte and becomes a gamete. During this process, the first 442 amino acids (aa) of the Pfs230 molecule are cleaved and the remaining Pfs230 is exposed on the surface of gamete [2]. While the biological role of Pfs230 in *P. falciparum* is not fully understood, it has been shown that Pfs230 forms a multimeric protein complex with Pfs48/45 (another TBV candidate) and *P. falciparum* LCCL (Limulus clotting factor C, the cochlear protein Coch-5b2, and the late gestation lung protein Lgl1) domain-containing proteins (PfCCp) [4]. In addition, the disruption of Pfs230 gene resulted in >90% reduction in oocyst numbers per mosquito compared to that in wild type parasites [5]. A study with *P230* gene disrupted rodent malaria parasite *P. berghei* indicated that P230 played an important role in male gamete fertility [6].

Quakyi et al. identified Pfs230 as a TBV candidate in 1987 [7], and since then multiple investigators have successfully produced Pfs230-based vaccines which induced “functional”

antibodies in animal models. Throughout the paper, the term of “functional” antibody means that antibody prevents oocyst formation in mosquitoes judged by a standard membrane-feeding assay (SMFA) and/or a direct membrane-feeding assay (DMFA). The epitope(s), which is recognized by the “functional” antibody, is called transmission-reducing epitope, TR epitope, in this manuscript (we don’t discuss whether the TR epitope has any essential function in the biology of mosquito infection). Previous studies include: mice or rabbits immunized with recombinant Pfs230 protein fragments produced using a variety of expression systems, *Escherichia coli* [8,9], plant *(Nicotiana benthamian)* [10], wheat germ cell-free system (WGCFS) [11,12], *Pichia pastoris* [13] and baculovirus [14]. In addition to the recombinant protein constructs, immunization with recombinant chimpanzee adenovirus 63 (ChAd63) expressing a part of Pfs230 molecule, followed by modified vaccinia virus Ankara (MVA), also induced functional antibodies in mice [15]. Furthermore, a Phase 1 human clinical trial with *P. pastoris* expressed Pfs230 conjugated with *Pseudomonas aeruginosa* ExoProtein A (EPA) has been conducted in the USA and Mali using Alhydrogel adjuvant (ClinicalTrial.gov Identifier: NCT02334462), and another trial with AS01 adjuvant is underway in Malian adults (ClinicalTrial.gov Identifier: NCT02942277). While the complete results of those human trials have not been published, the investigators reported a promising induction of antibodies that block in the SMFA from the first study [13]. In addition to the encouraging results in vaccinated animals and humans, immuno-epidemiology studies suggest that anti-Pfs230 immunity could be boosted by natural infections. Multiple cohort studies have shown that anti-Pfs230 antibody titers generally increase with age [16], and affinity purified anti-Pfs230-specific IgGs from naturally infected individuals significantly reduced oocyst density in SMFA [17].

These studies strongly support the rationale for Pfs230 as a promising TBV candidate antigen; however, the size and cysteine-rich nature of the molecule have hampered its production as a full-length antigen. Analysis of Pfs230 sequences suggests that it is comprised of repeated domains characteristic of 6-cys family proteins [18]. These predictions suggest that the Pfs230 molecule consists of 14 such 6-cysteine domains, or cysteine motif (CM) domain with each domain containing 4–6 cysteine residues [19,20]. An early study conducted in 1995 compared six different constructs spanning nearly 80% of the Pfs230, all of which were expressed in *E. coli* as fusion protein fragments with maltose-binding protein (MBP) [8]. In that study, only antibodies against r230/MBP.C (aa 443–1132) protein fragment were associated with consistent reductions in oocyst density in three independent SMFA. While informative, the 1995 study [8] may be limited by the ability of *E. coli*-based system to express properly folded cysteine-rich domain proteins. However, since then, all other studies [10–15] have focused on only 1/3 of the Pfs230 molecule (i.e., Pfs230C: aa 443–1132), and no further evaluation has been reported for other regions of Pfs230. Recently, the WGCFS has been used to express malaria recombinant proteins of native conformations for a variety of malaria vaccine candidates at different life stages and these proteins have successfully induced functional antibodies in animal models [12,21,22].

In this study, we took a comprehensive approach to analyze Pfs230 protein domains by taking advantage of the WGCFS. We systematically produced single-, two- and four-CM-domain constructs (a total of 27 protein fragments), which covered all 14 CM domains, to localize TR epitopes within the whole Pfs230 molecule. In addition, two more constructs

within the aa 443–1132 region, and one construct covering the cleavage site (aa 22–588) were produced. Antisera against individual constructs were generated in mice, and reactivity to native Pfs230 protein was evaluated by Western blot and immunofluorescence assay (IFA). Finally, the functionality of induced antibodies was assessed by SMFA. Our results indicate that N-terminal CM domain 1 could be the only region which is responsible for inducing transmission-reducing antibodies. While Pfs230 fragments which are expressed by systems other than WGCFS could reveal other new TR epitopes out-side of CM domain 1, unless such data are shown, it is reasonable to focus on CM1 region for the future Pfs230-based vaccine development.

2. Materials and methods

2.1. Design and expression of P. falciparum Pfs230 recombinant protein fragments using WGCFS

Amino acid (aa) sequence of Pfs230 (PF3D7_0209000) was obtained from the PlasmoDB database. To identify all possible TBV candidate domains, we systematically produced in the WGCFS either single-CM-domain (a total of 14), two-CM-domain (7) or four-CM-domain (6) recombinant protein fragments based on the proposed domain structures of Pfs230 [20] (Fig. 1). In addition, two more constructs, which covered previously published regions [13,14], and an N-terminal prodomain construct (aa 22–588) spanning the natural cleavage site (between aa 442–443) of Pfs230, were produced. In total, 30 Pfs230 constructs were designed as shown in Supplementary Table 1 and Fig. 1. Among the largest gene fragments (TBV01, 02, 03, 04, 29, 30 and 31), the nucleotide sequences were codon optimized for expression in wheat (Gen-Script, Piscataway, NJ), and an Xhol restriction site with start codon at the N-terminus and the hexa histidine-tag (His-tag) followed by the stop codon and Notl site were introduced at the C-terminus. Each synthetic gene was cloned between Xho I and Not I sites of the pEU-E01-MCS plasmid, which is designed specifically for the WGCFS (CellFree Sciences, Matsuyama, Japan). The other gene fragments were subcloned from these synthetic genes. As a negative control, N-terminal His-tagged GST (HisGST) was produced using pEU-E01-GST vector without an additional fusion partner. We used the WGCFS to synthesize recombinant protein fragments as described [21,23]. Pfs230 and HisGST constructs were affinity purified using Ni-Sepharose columns (GE Healthcare, Camarillo, CA), and subsequently eluted by imidazole. In case the solubility of the purified recombinant protein fragment was low, the construct was synthesized in the presence of 0.05% polyoxyethylene(23) lauryl ether (Brij35) (Wako Pure Chemical, Osaka, Japan) non-ionic detergent in the WGCFS [24]. The recombinant protein fragments were analyzed by a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and the bands were visualized with Coomassie brilliant blue R-250. The recombinant proteins were stored in aliquots at −80 °C until used.

2.2. Generation of mouse anti-Pfs230 antiserum and ELISA

To generate antisera for each construct, a group of seven ICR female mice 8-weeks-old were immunized with 20 μg of purified recombinant Pfs230 protein fragment with Freund's complete adjuvant intraperitoneally on day 0 followed by 20 μg of the protein with Freund's incomplete adjuvant on day 21 (Kitayama Labes, Ina, Japan). Antisera were collected 21

days after the last immunization. The animal work was conducted by Kitayama Labes (Ina, Japan) in compliance with the guidelines based on “Charter for Laboratory Animal Welfare” (Japanese Society for Laboratory Animal Resources). For each group, antibody levels against the corresponding immunogen were determined individually by enzyme-linked immunosorbent assay (ELISA). The basic methodology of the ELISA has been described [11,12]. Briefly, serum samples were plated in duplicate at a starting dilution of 1:1000 and titrated in 10-fold dilutions. Reciprocal serum dilutions that gave a mean absorbance value of 0.5 at 415 nm were determined as the endpoint titers. For the following assays, a pool of sera from four mice with higher antibody titers in each group was generated.

IgG subclass ELISA was performed as described previously [14] with a small modification; all IgG samples were diluted at OD = 1.5 for total IgG, instead of 1 ELISA unit.

2.3. Western blot analysis

Proteins of the cultured stage V gametocytes of the *P. falciparum* NF54 line were extracted in non-reducing SDS-PAGE loading buffer and boiled at 98 °C for 3 min, and the extract from approximately 10^5 gametocytes per lane was subjected to electrophoresis on a 7.5% polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were then transferred to a 0.2-μm polyvinylidene fluoride (PVDF) membrane (ATTO). The proteins were immunostained with pooled immune serum as the primary antibody (1:500 dilution). The membranes were then probed by HRP-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific, Waltham, MA) and visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA) on an ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare). In the case of positive control lanes, rabbit anti-Pfs230C (aa 443–1132) antibody was used and probed by HRP-conjugated goat anti-rabbit IgG antibody (Thermo Fisher Scientific) as reported [11]. The relative molecular masses of the proteins were estimated with reference to HiMark™ Pre-Stained Protein Standard (ThermoFisher Scientific). The human plasma and red blood cells used for the gametocyte cultures at Ehime University were obtained from the Japanese Red Cross Society.

2.4. Immunofluorescence assay (IFA)

An indirect IFA was performed with cultured gametocytes of the *P. falciparum* NF54 line. Air-dried thin smears of the parasites were prepared on glass slides, fixed with ice-cold acetone for 3 min and stored at −80 °C until use. The smears were thawed, blocked with phosphate buffered saline (PBS) containing 5% nonfat milk (PBS milk) at 37 °C for 30 min. They were then incubated with both anti-Pfs230 mouse pooled serum (1:200 dilution) and rabbit anti-Pfs230C immune serum (1:500 dilution) [11] as gametocyte marker at 37 °C for 1 h, followed by incubation with a mixture of Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) (1:500) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (Invitrogen) (1:500) as secondary antibodies at 37 °C for 30 min. Slides were mounted in ProLong Gold Antifade reagent (Invitrogen) and observed using a fluorescence microscope (Axio observer z1, Carl Zeiss, Oberkochen, Germany).

2.5. IgG purification and SMFA

Total IgGs from pooled serum samples were purified using Protein G columns (GE Healthcare) according to the manufacturer's instructions, and adjusted to a final concentration of 12–20 mg/ml in PBS.

The standardized methodology for performing the SMFA was described previously [25]. Briefly, 16–18 day old gametocyte cultures of the *P. falciparum* NF54 line were mixed with test IgGs at 1.5 mg/ml, and the final mixture was immediately fed to ~50 female *Anopheles stephensi* mosquitoes through a membrane-feeding apparatus. All feeding experiments were performed with human complement. Mosquitoes were kept for eight days and dissected (n = 20 per group) to enumerate the oocysts in the midgut. Only midguts from mosquitoes with any eggs in their ovaries at the time of dissection were analyzed. The human serum and red blood cells used for the gametocyte cultures and feeding experiments performed at the National Institute of Allergy and Infectious Diseases were purchased from Interstate Blood Bank (Memphis, TN).

2.6. Statistical analysis

The best estimate of % inhibition in oocyst density (% transmission-reducing activity, %TRA), the 95% confidence intervals (95%CI), and p-values from single or multiple feeds were calculated using a zero-inflated negative binomial random effects model (ZINB model) described previously [26]. IgG subclass ratios between two groups were compared by a Mann-Whitney test. All statistical tests were performed in R (version 3.4.1) or Prism 7 (GraphPad Software), and p-values <0.05 were considered significant.

3. Results

3.1. Design and expression of Pfs230 constructs

In order to identify location of TR epitopes within the whole Pfs230 molecule, we designed 30 Pfs230 constructs as summarized in Supplementary Table 1 based on the proposed domain structures of Pfs230 [20] (Fig. 1). Although 13 out of 30 Pfs230 fragments were efficiently expressed with the WGCFS, the other 17 Pfs230 fragments required addition of Brij35 detergent in WGCFS to increase their solubility [24] (Supplementary Table 1). Finally, all the Ni-affinity-purified recombinant protein fragments, including a negative control HisGST, were successfully obtained at a yield of over 300 μg each, which was sufficient to immunize seven mice twice (Fig. 2).

3.2. Reactivity of mouse antibodies against immunogen and native proteins

To generate antibodies against each construct, ICR mice (n = 7 per group) were immunized with each Pfs230 protein fragment or the control protein HisGST. On day 42, antibody titer in each serum sample against the immunogen was determined by ELISA. Median IgG ELISA titer in each group ranged from $10^{3.2}$ to $10^{5.7}$ (Supplementary Table 1). These results suggest that all the WGCFS produced recombinant protein fragments were immunogenic in mice.

To systematically identify domains with TR epitopes in the whole Pfs230 molecule, we selected four mice with comparatively higher titers of antibodies against the immunogen in each group to make a serum pool. Average ELISA titers among the pooled mouse antiserum in each group were distributed between $10^{3.9}$ and $10^{5.8}$ (Supplementary Table 1).

Reactivity and specificity of each pooled mouse antiserum sample were first evaluated by non-reducing Western blot analysis using gametocyte extracts of the *P. falciparum* NF54 line as an antigen. In each experiment, anti-HisGST antibody was used as a negative control (TBV28), and anti-TBV27 mouse antibody was included as a positive control, as the same construct (called "Pfs230C") specifically recognized Pfs230 and induced functional antibodies in the previous studies [11,12]. Specific bands with the expected mobility of native Pfs230 protein around 300 kDa were detected with anti-TBV27 mouse antibody (labelled as 27 in Fig. 3) and another positive control rabbit anti-Pfs230C antibody (Fig. 3, lane R2 in each blot) [11,12]. Most of the mouse antibodies against each recombinant Pfs230 fragment specifically recognized the native Pfs230 with variable band intensities (Fig. 3). Among them, relatively higher reactivities were observed by antibodies against TBV01, 05, 12, 18, 26, 27, and 31 followed by moderate reactivities against TBV02, 03, 07, 08, 13, 15, and 23. The other anti-Pfs230 antibodies showed faint (TBV04, 06, 10, 11, 14, 16, 17, 19, 20, 22, 25, 29, and 30) or negligible (TBV09, 21, and 24) reactivities. Anti-HisGST negative control antibody did not recognize Pfs230 (Fig. 3, lane 28 in each blot). These results suggest that most of the recombinant Pfs230 fragments prepared by the WGCFS at least in part retained native epitopes.

Next, IFA with mature gametocytes was performed to test if the antisera could recognize native Pfs230 protein on the parasite. The native Pfs230 protein on the gametocytes was labelled with positive control rabbit anti-Pfs230C antibody (Fig. 4, R2). Around half of the mouse antibodies against recombinant Pfs230 fragments recognized parasites, presumably native Pfs230 protein (based on the single and predicted size of band seen in the Western blot analysis, Fig. 3), with variable fluorescent intensities (Fig. 4, upper panels). Among them, relatively higher intensities were observed by antibodies against TBV01, 05, 08, 12, 26, 27, and 31 followed by moderate reactivities against TBV02, 03, 07, 11, 13, 18, 20, 23, and 30. The other anti-Pfs230 antibodies showed negligible reactivities. Anti-HisGST negative control antibody did not recognize Pfs230 (Fig. 4, TBV28).

Taken together, 17 out of 30 anti-Pfs230 fragment mouse antibodies spanning whole Pfs230 domains react on native Pfs230 either by Western blot or IFA (summarized in Fig. 1 and Supplementary Table 1). Especially, higher reactivities against native Pfs230 determined with both Western blot and IFA were shown by TBV01, 05, 12, 26, 27 and 31.

3.3. SMFA evaluation for anti-Pfs230 antibodies against all constructs

Total IgG against each Pfs230 construct was tested at a concentration of 1.5 mg/ml, with human complement, in two independent assays. In each SMFA, anti-HisGST IgG (TBV28) was used as a negative control, and anti-TBV27 IgG was included as a positive control. The original feeding data in each experiment are available in Supplementary Table 2. In addition to the positive control (anti-TBV27 IgG, targeting CM_1–3), IgGs against all other constructs which contained CM domain 1 region (TBV01, CM_1–4; TBV05, CM_1–2;

TBV12, CM_1; TBV26, CM_1) showed significant inhibitions (>94% TRA, $p < 0.001$ for all). In contrast, IgGs against other protein fragments, which did not include CM domain 1, showed insignificant inhibitions (<33% TRA, $p \geq 0.150$). The 5 functional IgGs were further evaluated at 4 different concentrations in SMFA, and all of them showed dose-dependent inhibitions (Fig. 5).

All of the four constructs containing the CM7 region were able to induce antisera that were positive in both non-reducing Western blot (3 out of 4) and IFA (4 out of 4), while they did not induce SMFA activity, in contract to fragments with CM1. The difference in SMFA could be explained by the different characteristics, such as IgG subclass profile, of the induced antibodies. Therefore, IgG subclasses for the antibodies against constructs contained CM1 (5 IgGs) or CM7 (4 IgGs) were determined (Fig. 6). As shown in Fig. 6, there was no obvious difference between CM1- and CM7-containing constructs in terms of their IgG subclass profiles. When the IgG2/IgG 1 ratio (the sum of the OD values (IgG2a + IgG2b + IgG2c) divided by the OD value of IgG1) was calculated for individual IgGs, there was no significant difference in the ratio between the two groups of IgGs ($p = 0.56$).

In addition to the above constructs which covered the entire region of the cleaved Pfs230 (aa 443–3135) [8], we also evaluated mouse IgG against prodomain (aa 22–588) of the Pfs230 recombinant protein fragment. This was designed to determine whether (a subset of) anti-prodomain antibodies might interfere with the natural cleavage process, resulting in inhibition of oocyst formation. Despite the relatively higher reactivities on native Pfs230 by both Western blot (Fig. 3A) and IFA (Fig. 4), the total IgG sample did not show any inhibition in SMFA (−6.2% TRA, $p = 0.877$).

4. Discussion

In this study, we systematically evaluated entire domains of Pfs230 using recombinant protein fragments produced by WGCFS. Twenty-seven constructs, which contained either single-CM-domain (a total of 14 protein fragments), two-CM-domain (a total of 7) or four-CM-domain (a total of 6), two more constructs which covered previously published regions [13,14] and the other N-terminal prodomain construct of Pfs230 were generated in this study. Out of the 30, only five protein fragments with CM domain 1 (aa 589–730)) [20] (Supplementary Table 1 and Fig. 1) induced functional antibodies as determined by SMFA (>94% TRA at 1.5 mg/ml). Antibodies against any other constructs that did not include the CM domain 1 showed insignificant inhibitions in SMFA (<33%TRA at 1.5 mg/ml). The results suggest that all, or at least majority of, TR epitopes in Pfs230 molecule are located in the N-terminal region.

Thirteen out of 30 Pfs230 fragments were efficiently expressed by the WGCFS. These data are consistent with previous reports of our successful application of the WGCFS to *Plasmodium* recombinant protein expression [21]. The remaining 17 Pfs230 fragments were also successfully expressed in sufficient quantities via the addition of Brij35 detergent to increase solubility [24]. These results continue to support the utility of the WGCFS to generate a great variety of difficult to express proteins of interest as vaccine candidate

antigens, such as Pfs230. This system has advantages for such research in that soluble proteins can be produced from a variety of constructs in a very short time.

There are several possible explanations to account for the lack of SMFA activity of antibodies against the constructs not including the CM domain 1. We recognize that the generation of a native conformation in the expressed protein is a requirement for induction of sera that are functionally active in SMFA [2,3]. Out of the 30 constructs produced in this study, antibodies against 43% (13/30) of constructs showed no or very weak reactivity against native Pfs230 proteins judged by Western blot and IFA (Figs. 3 and 4), while all of the 30 constructs elicited antisera which recognized the corresponding immunogens judged by ELISA (Supplementary Table 1). Therefore, it is reasonable to speculate that those constructs, both Western- and IFA-negative, may not been expressed in the native conformations. On the other hand, the remaining 57% (17/30) of constructs could induce antibodies which reacted to the Pfs230 proteins in Western blot or IFA. However, out of them, only 5 constructs contained the CM domain 1 elicited SMFA-active antibodies. As described above, no antibodies showed an intermediate level of inhibition in SMFA at 1.5 mg/ml; rather, they exhibited either strong (>94% TRA), or minimal (<33% TRA) inhibition. The results suggest that there was a fundamental difference(s) between SMFA-positive and SMFA-negative constructs. Since all constructs with CM domain 1 could induce functional antibodies, it seems most likely that CM domain 1 is the only domain which satisfies both of the following criteria; namely, expressed in a native conformation and contains TR epitopes. We speculate that CM7 meets one criterion (correct conformation), but not the other (contains TR epitopes). For some antibodies, such as anti-TBV11 and anti-TBV15 antibodies, we observed discrepancies between Western blot and IFA (i.e., reactive in non-reducing Western blot but non-reactive in IFA, or vice versa). These inconsistency may be explained (at least a part) by the different conditions in the two assays; namely, in particular, gametocyte proteins in Western blot analysis were treated with SDS, but not for the IFA. In any case, our analysis can be interpreted as covering the entire extent of the Pfs230 protein with WGSFS expressed construct where there is evidence of appropriate native folding based on the induced sera.

We cannot rule out the possibility that one might figure out other new TR epitopes outside of CM domain 1, when other expression systems are utilized to produce other constructs. However, the current study, in conjunction with the previous reports, suggest that the design of Pfs230-based vaccines should focus on the CM1 domain. Due to the relative small size and relative simplicity of the protein fold of the CM1 region, this greatly simplifies the future development of the malaria transmission-blocking vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CM	cysteine motif
DMFA	direct membrane-feeding assay
IFA	Immunofluorescence assay
SMFA	standard membrane-feeding assay
TBV	transmission-blocking vaccine
TRA	transmission-reducing activity
TR epitope	transmission-reducing epitope
WGCFS	wheat germ cell-free system

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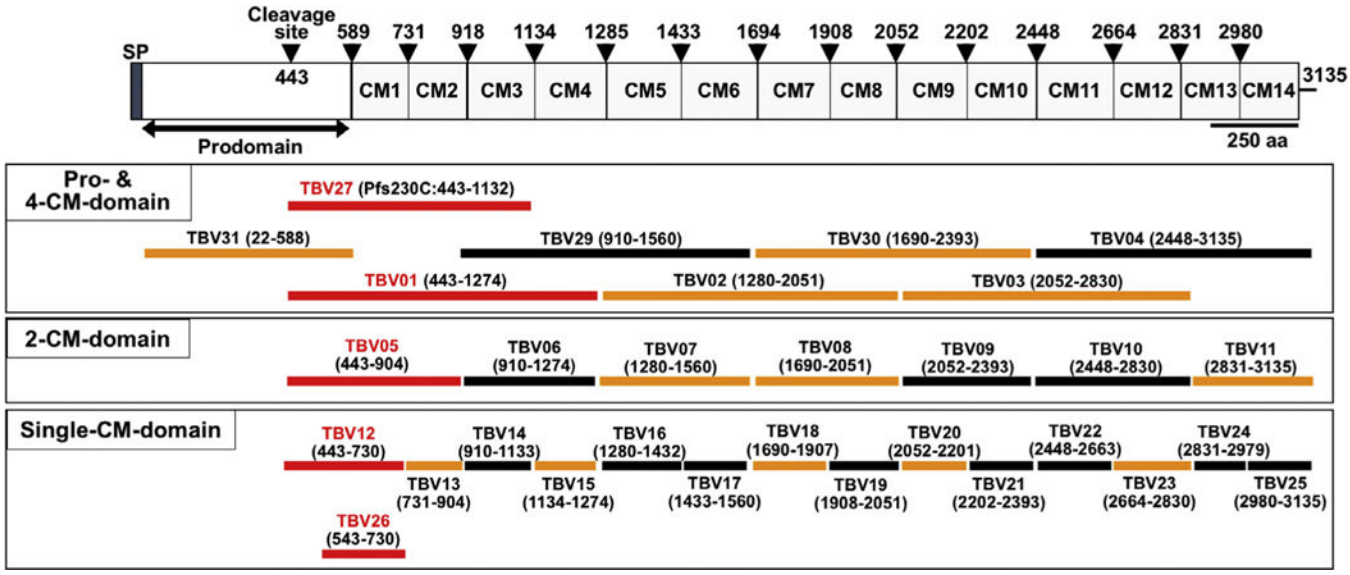

Fig. 1.

Pfs230 domain structure and design of constructs. SP represents a signal peptide. The cleavage site at amino acid (aa) 443 represents the site at which cleavage of Pfs230 occurs during gamete formation. The region comprising aa 22–588 refers to the prodomain. CM1 through CM14 represent cysteine motif domains (CM) as described by Gerloff et al. [20]. Amino acid positions (arrowheads) represent the start of each CM. Pfs230 constructs are classified into 3 groups, i.e., “Pro- & 4-CM-domain”, “2-CM- domain”, and “Single-CM-domain”. Antibodies against constructs indicated in red bars showed significant transmission-reducing activities (TRA) with positive reactivities on both Western blot and IFA. Antibodies against constructs indicated in orange bars showed positive reactivities on Western blot and/or IFA but negative TRA. Detailed information about each construct is presented in Supplementary Table 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

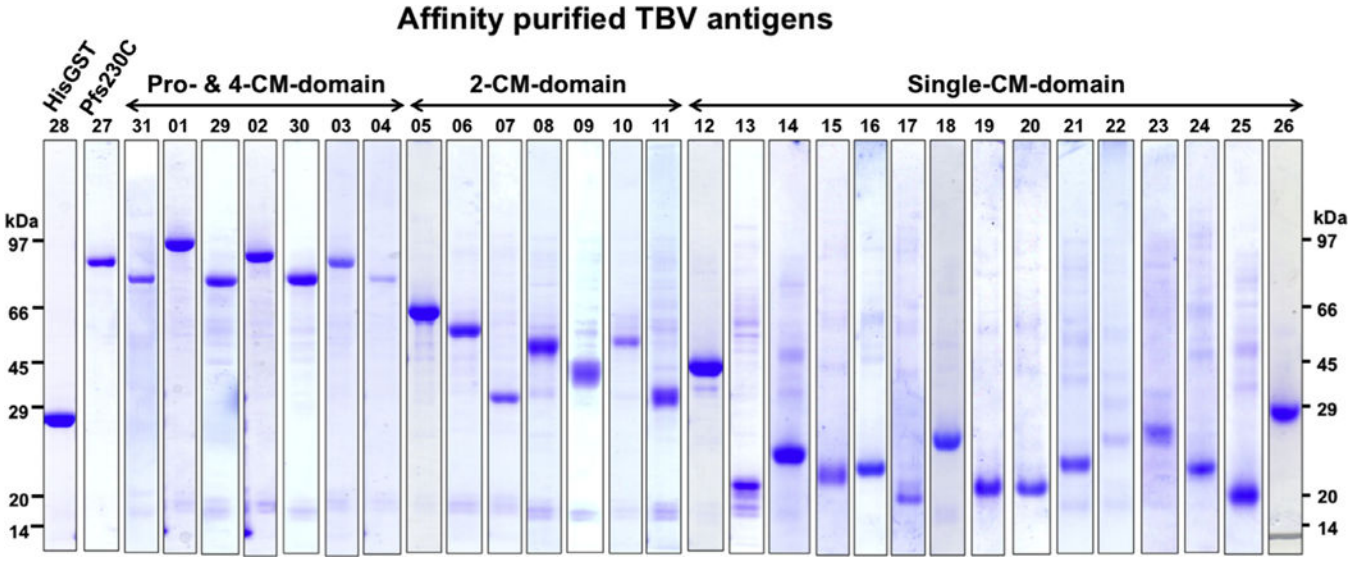

Fig. 2.

SDS-PAGE analysis of the Ni-affinity-purified recombinant Pfs230 fragments expressed with WGCFS. Ni-affinity-purified recombinant Pfs230 fragments expressed in the wheat germ cell-free system were separated on SDS-12.5% polyacrylamide gel under reducing condition and stained with Coomassie brilliant blue. Each number on the top of each lane represents antigen name, for example 31 indicates TBV31, etc. Pfs230 constructs are classified into 3 groups, i.e. “Pro- & 4-CM-domain”, “2-CM-domain”, and “Single-CM-domain”. TBV28 represents HisGST as a negative control. TBV27 represents published Pfs230C fragment [12] which spans CM1 to CM3 and serves as a positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

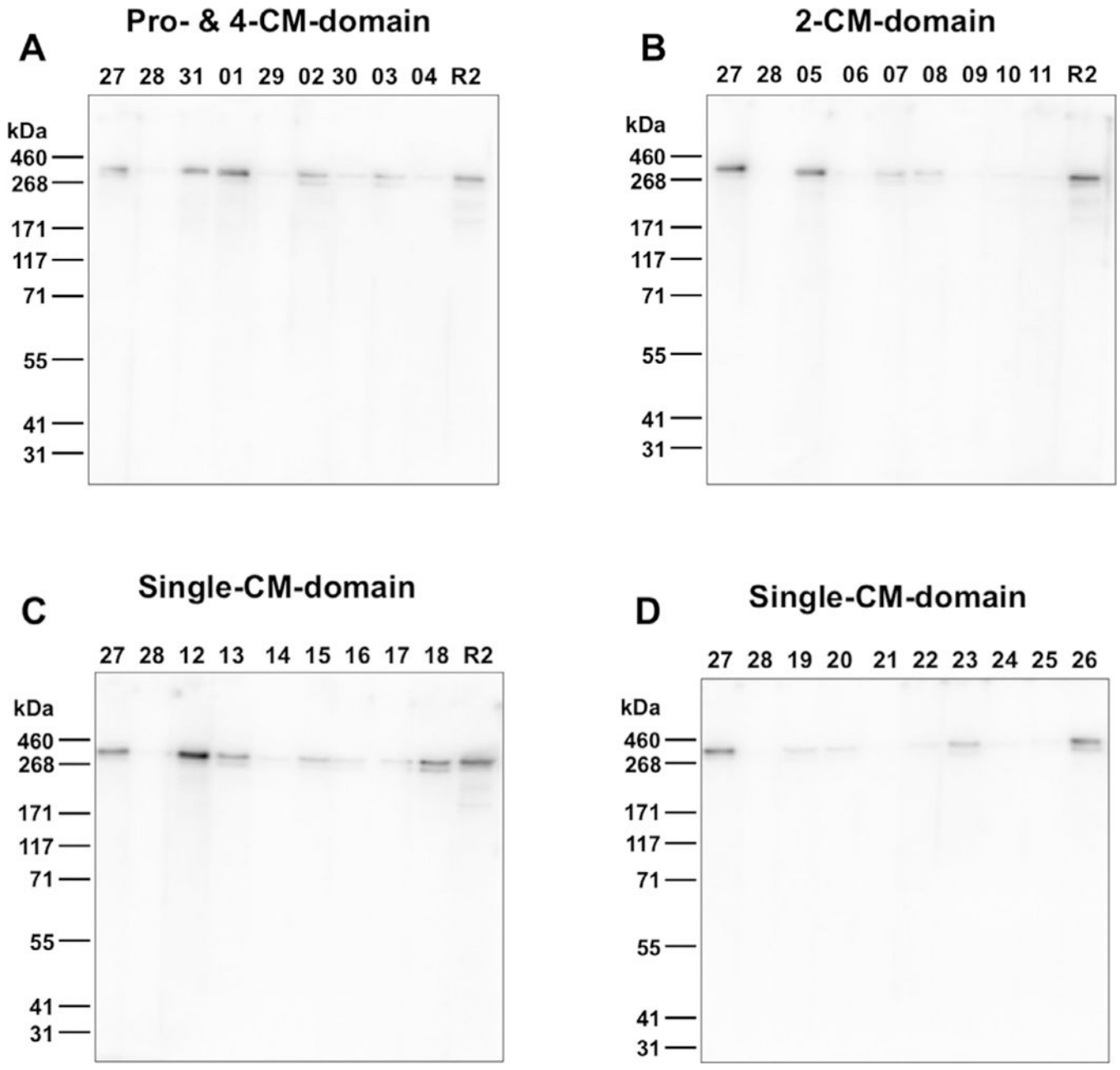

Fig. 3.

Western blot analysis using antisera against different fragments of Pfs230. Extracts prepared from gametocytes of the *P. falciparum* NF54 line were separated on SDS-7.5% polyacrylamide gels under non-reducing condition and transferred onto PVDF membrane. Proteins on PVDF membranes were immunostained with either rabbit anti-Pfs230C positive control antibodies (lanes R2 in each panel) or the corresponding anti-Pfs230 fragment mouse antibodies. Each number on the top of each lane represents the TBV fragment used for induction of antisera, for example 31 indicates anti-TBV31 antibody, etc. Western blot data are classified into 3 groups, i.e. “Pro- & 4-CM-domain” presented in A, “2-CM-domain” presented in B, and “Single-CM-domain” presented in C and D. TBV28 represents

anti-HisGST mouse antibody as a negative control. TBV27 represents anti-Pfs230C mouse antibody as positive control [12]. The relative molecular masses of the proteins were estimated with reference to HiMark™ Pre-Stained Protein Standard.

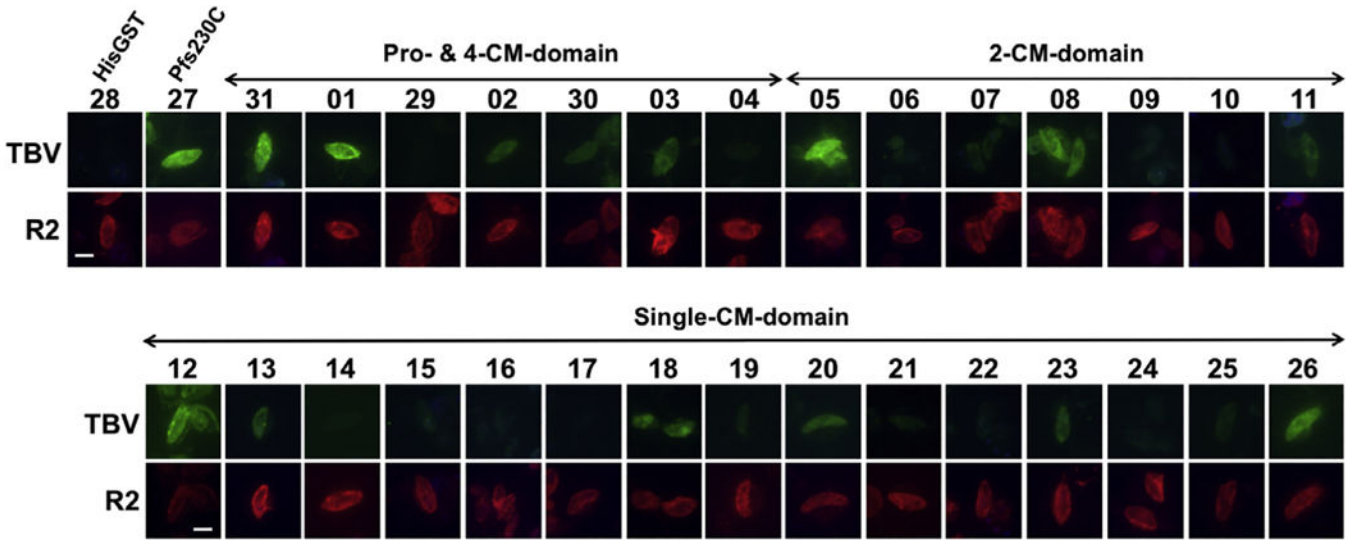

Fig. 4.

Reactivity of antisera against Pfs230 fragments in IFA Samples prepared from gametocytes of the *P. falciparum* NF54 line were double stained with the mouse antisera indicated on the upper side of the panels and rabbit anti-Pfs230C positive control antibodies (R2, lower images in each panel). Immunostained images were visualized with Alexa Fluor 488-conjugated goat anti-mouse IgG (green) and Alexa Fluor 546-conjugated goat anti-rabbit IgG (red). TBV28 represents anti-HisGST mouse antibody as a negative control. TBV27 represents anti-Pfs230C mouse antibody as positive control [12]. Scale bars, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

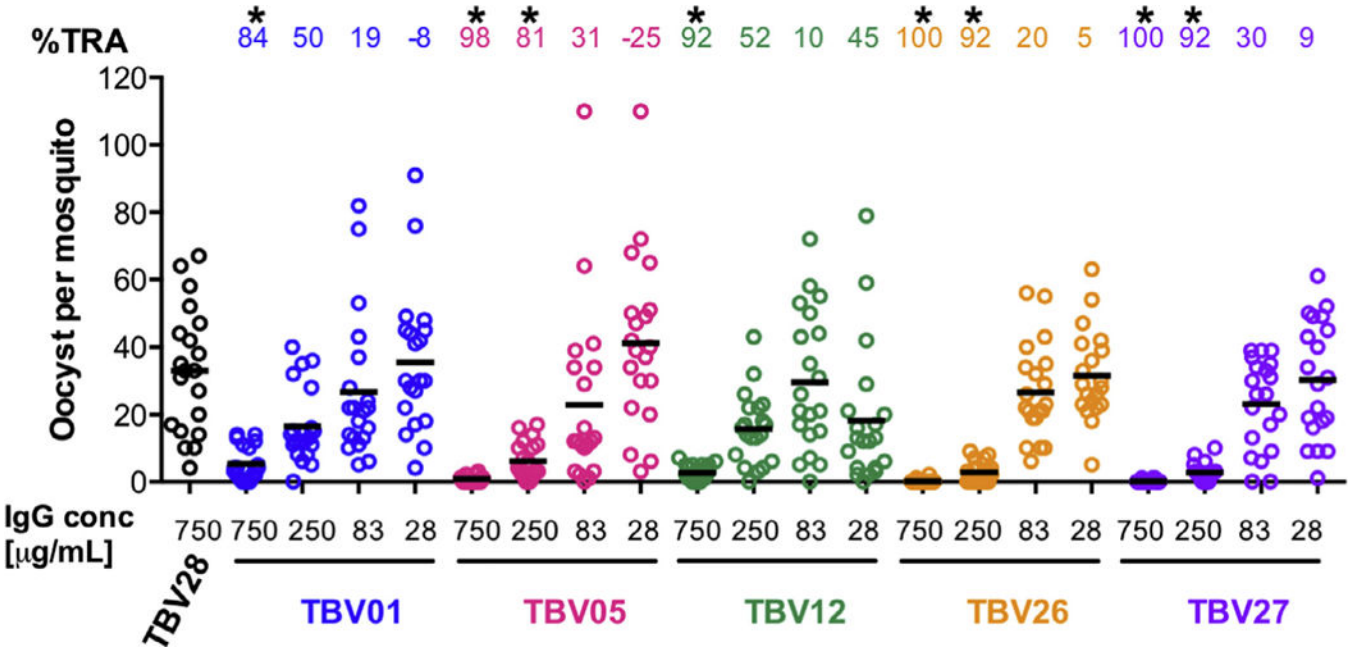

Fig. 5.

Dose response of functional antibodies in SMFA. The negative control (TBV28) and five SMFA-positive (TBV01, TBV05, TBV12, TBV26 and TBV27) IgGs were tested at the indicated concentrations in SMFA. Oocyst number in individual mosquitoes (open circles), group mean (bar), % TRA values (compared to TBV28 data), and significance of% TRA (*, $P < 0.001$) are shown.

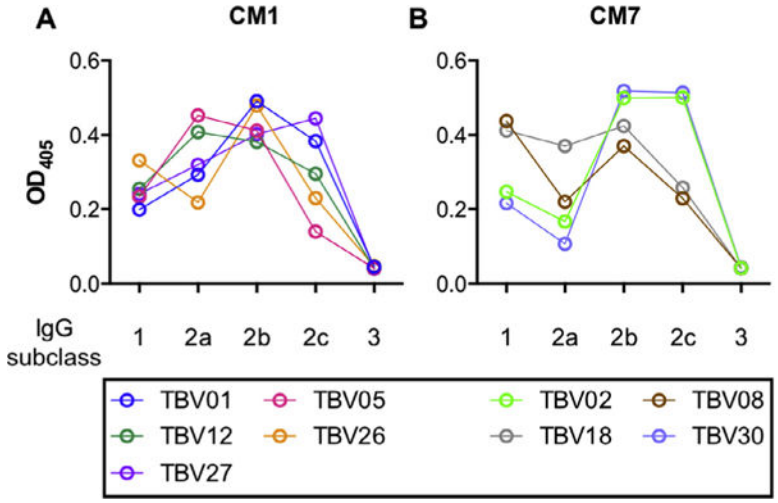

Fig. 6.

IgG subclass profile for antibodies against CM1- or CM7-containing constructs. IgGs against five CMl-containing fragments (TBV01, TBV05, TBV12, TBV26 and TBV27) were tested using ELISA plates coated with TBV26-GST fusion antigen, and IgGs against four CM7-containng fragments (TBV02, TBV08, TBV18 and TBV30) were tested with TBV18-GST antigen. All IgGs were diluted at OD = 1.5 for total IgG, and then tested with anti-mouse IgG subclass specific secondary antibodies.