

Recombinant Pfs25 Protein of *Plasmodium falciparum* Elicits Malaria Transmission-blocking Immunity in Experimental Animals

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Summary

Pfs25 is a sexual stage antigen of *Plasmodium falciparum* that is expressed on the surface of zygote and ookinete forms of the parasite. Monoclonal antibodies directed against native Pfs25 can block completely the development of *P. falciparum* oocysts in the midgut of the mosquito vector. Thus, this 25-kD protein is a potential vaccine candidate for eliciting transmission-blocking immunity in inhabitants of malaria endemic regions. We have synthesized, by secretion from yeast, a polypeptide analogue of Pfs25 that reacts with conformation-dependent monoclonal antibodies, and elicits transmission-blocking antibodies when used to immunize mice and monkeys in conjunction with a muramyl tripeptide adjuvant. Our results suggest the further evaluation of recombinant DNA-derived Pfs25 in transmission-blocking vaccination studies in humans.

The increasing resistance to chemotherapy of the parasites responsible for human malaria has reaffirmed the requirement for an effective malaria vaccine. Subunit vaccine strategies using either peptide synthesis or recombinant DNA technologies have been focused primarily on the immunization of the human host against asexual forms of the parasite. For example, well-studied vaccination approaches have targeted the induction of neutralizing responses against the sporozoite form of the parasite that is injected by the mosquito vector during a blood meal. Also, merozoite and erythrocytic-stage antigens have been considered as immunogens for the suppression of parasitemia in susceptible individuals. An alternative approach for the control of endemic malaria in developing countries is that of transmission-blocking vaccination. Here, it has been proposed that such a strategy could prevent the transmission of viable sexual forms of the parasite from humans to mosquitoes. Foremost among transmission-blocking vaccine candidates is the *Plasmodium falciparum* surface protein Pfs25. This 25-kD polypeptide is expressed on the surface of zygote and ookinete forms of the parasite as they develop in the midgut of the mosquito vector (1). mAbs to Pfs25 have been shown to block fully the development of sexual stages of the parasite (1, 2), and vaccinia virus recombinants that express Pfs25 can induce transmission-blocking antibodies in MHC-disparate congenic mice (3). Such studies have suggested that the production of large quantities of synthetic Pfs25 peptides, or recombinant Pfs25 protein, together with recreation of the appropriate conformation of B cell epi-

topes for immunological activity in vivo are required for the development of Pfs25 as a human vaccine. Here, we show that an analogue of Pfs25, synthesized by secretion from yeast, reacts with conformation-dependent mAbs, and can elicit transmission-blocking antibodies when used to immunize mice and monkeys in conjunction with a muramyl tripeptide (MTP) adjuvant.

Materials and Methods

DNA Manipulations. Oligonucleotides were synthesized by the phosphoramidite method using DNA synthesizers (380B; Applied Biosystems, Inc., Foster City, CA). Gene assembly and site-directed mutagenesis were performed using standard procedures (4).

Yeast Expression and Protein Purification. The synthetic Pfs25 genes were cloned into the yeast vector pBS24 (5) and the resulting plasmids were used to transform yeast strain AB110 (6). Pfs25-B was purified from yeast supernatants subsequent to removal of yeast cells by continuous flow centrifugation. Supernatants were concentrated and desalted by tangential flow ultrafiltration over a 5-kD membrane. Initial purification was achieved by ion-exchange chromatography on Fast Flow Q resin (Pharmacia Fine Chemicals, Piscataway, NJ), equilibrated with 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, using a 0–0.3 M NaCl gradient for elution. The elution profile was monitored at A₂₈₀, and the major peak was confirmed as Pfs25-B by SDS-PAGE. Fractions that contained Pfs25-B were pooled, concentrated by ultrafiltration to 10–20 mg/ml, and separated by gel filtration on S-100 HR Sepharose (Pharmacia Fine Chemicals). The column was developed in 2× PBS and monitored in an identical manner to the ion-exchange purification step. The

peak protein fractions were pooled, concentrated, desalted, and the material lyophilized and stored at 4°C.

Immunizations. In the Freund's adjuvant group, mice were immunized three times at 3-wk intervals with 50 µg of Pfs25-B in a final volume of 100 µl, as described previously (7). In the MF59 adjuvant groups, mice were immunized, using the same protocol with Pfs25-B, falc 2.3 (7), hEGF (8), or hSOD (9) (50 µg/injection). Animals were bled 1 wk after the final immunization, and antibody titers were measured in the ELISA format. Monkeys were immunized with Pfs25-B and MF59, or MF59 alone using the same protocol, except that injections were carried out at 4-wk intervals. Immunized and control animals were bled at 2-wk intervals for ELISAs, and transmission-blocking activities were measured at weeks 12 and 22.

ELISA. ELISAs were performed as described previously (7). Titers were calculated at 0.2 absorbance units using a V_{max} microtiter plate reader (Molecular Devices, Palo Alto, CA) programmed to read at 650 nm with subtraction of the 490-nm reading.

Antibodies. mAb 4B7 was produced by fusion of spleen cells from a BALB/c mouse immunized twice with recombinant vaccinia virus that were expressing Pfs25, and boosted with whole *P. falciparum* gametes. Supernatants from the fusion were screened for specificity to Pfs25 by immunoblot of reduced Pfs25-B (D. C. Kaslow, unpublished observations). Antisera P7 was produced by pooling sera from mice immunized individually with the peptides SLKCLKENETCKAVD, LKENETCKAVDGIYK, KAVDGIYKCDCKDGF, GIYKCDCKDGFIIIDN, and DCKDGFIIIDNESSI, which are derived from the fourth EGF-like domain of Pfs25.

Transmission-blocking Assays. Murine or monkey sera were assayed for transmission-blocking activity as described previously (2). Briefly, sera or mAb ascites fluid were mixed in a 1:1 ratio with erythrocyte cultures that contained *P. falciparum* gametocytes and the mixtures were fed to *Anopheles freeborni* mosquitoes using a membrane feeding apparatus. After 7 d, mosquitoes were dissected and examined for the presence of *P. falciparum* oocysts (2).

Results and Discussion

We chemically synthesized a gene for Pfs25 that encoded amino acids 22–190 of the natural 217–amino acid precursor protein (10), and thereby deleted the proposed NH₂-terminal

secretory signal sequence. Also deleted was the hydrophobic COOH-terminal region that has been proposed to serve as a signal for attachment of a glycosylphosphatidyl-inositol membrane anchor (10, 11). Expression and secretion from yeast of this Pfs25 extracellular domain was achieved by fusion of the synthetic gene to DNA sequences encoding the yeast α factor pheromone secretory signal/leader sequence (8). Transcription was driven by the glucose-regulated alcohol dehydrogenase 2/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAPDH) hybrid promoter described previously (12). A second gene was also constructed, in which the three remaining N-linked glycosylation sites at amino acid positions 112, 165, and 187 of the encoded protein (Fig. 1 A) were removed by Asn→Ala mutation and introduction of a termination codon after Glu188. The removal of glycosylation sites allowed the secretion of a homogeneous product that also contained all four epidermal growth factor (EGF)-like repeats described previously for Pfs25 (Fig. 1 A) (10). This protein, designated Pfs25-B, was selected for further study.

The secreted protein was purified (Fig. 1 B), and analyzed by immunoblotting using rabbit polyclonal antisera to Pfs25 or with the mAb 1C7 (10), a transmission-blocking mAb that had been shown previously to recognize natural Pfs25 only under nonreducing conditions. The purified protein was used to immunize mice and *Aotus trivirgatus* monkeys with the muramyl tripeptide (MTP) adjuvant MF59, an adjuvant that has been used previously to elicit high antibody titers in rodents and goats with other recombinant malaria proteins (7), and has also been demonstrated to be safe and efficacious in humans when used in conjunction with a yeast-derived HIV-1 envelope protein (13).

Humoral immune responses elicited in mice with both Freund's adjuvant and MF59 (Table 1), and in monkeys with MF59 (Fig. 2), were measured by ELISA. Also shown are the reactivities of antisera raised with recombinant Pfs25 against various control recombinant proteins from yeast. These include human superoxide dismutase (hSOD) (9), a circumsporozoite (CS) protein from *P. falciparum* (falc 2.3) (7), and

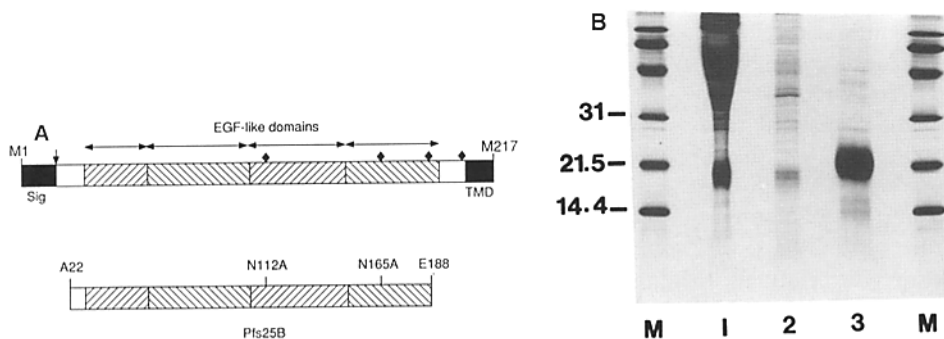


Figure 1. (A) Schematic representations of the natural 217-amino acid Pfs25 protein and the yeast recombinant Pfs25-B. Shown are the four regions with sequence homology to EGF (hatched and arrowed), potential N-linked glycosylation sites (◆), a putative signal peptide sequence (Sig), and consensus signal peptidase cleavage site (↓). Also shown is a hydrophobic transmembrane domain (TMD) that serves as an attachment signal for a glycosylphosphatidyl-inositol anchor (8, 9). Sites within Pfs25-B that were mutated to remove glycosylation sites are marked at Asn112 and Asn165. (B) Ex-

pression, secretion, and purification of Pfs25-B from yeast. Molecular mass markers are in kilodaltons (Bio-Rad Laboratories) (Lane M); TCA precipitate of media from recombinant yeast cells transformed with the plasmid pBS24-Pfs25-B (lane 1); pooled Pfs25-B fractions from chromatography on A-Sepharose Fast Flow (Pharmacia Fine Chemicals) (lane 2); pooled Pfs25-B fractions from a further purification step on Sephacryl S-100 HR (Pharmacia Fine Chemicals) (lane 3). Proteins were fractionated by 15% SDS-PAGE in the presence of 2-ME.

Table 1. Antibody Titers Developed in Mice by Immunization with Recombinant Pfs25-B and either Freund's (FCA/FIA) or MF59 Adjuvants

Antigen	Adjuvant	Assay protein			
		Pfs 25B	Falc 2.3	hEGF	hSOD
Pfs25-B	FCA/FIA	42,000	<100	<100	<100
Pfs25-B	MF59	31,625	<100	<100	<100
Falc 2.3	MF59	<100	20,200	<100	ND
hEGF	MF59	<100	<100	9,100	ND
hSOD	MF59	<100	ND	<100	18,750

human EGF (8, 14). In addition, sera from each immunized monkey (12-wk time point) reacted with native Pfs25 on non-reducing immunoblots (Fig. 3 A).

The ability of sera to block the formation of oocysts in *P. falciparum* gametocyte-fed mosquitoes was determined as described previously (2), and the results are shown in Table 2 A. Clearly, murine antisera against Pfs25-B from either Freund's or MF59-adjuvanted mice were able to dramatically reduce or even abolish the sexual development of the parasite within the mosquito midgut. Only one oocyst was detected in 37 mosquitoes dissected from the immune sera groups, whereas all 16 control, preimmune sera-fed mosquitoes developed oocysts, with an average number of eight per mosquito. This is comparable with results obtained using the transmission-blocking mAb 1D3. This mAb, at a dilution of 1:2 of ascites fluid, fully blocked oocyst development in all 16 mosquitoes tested (Table 2 A). Similarly, sera from two *A. trivigatus* monkeys were capable of blocking completely the development of oocysts in mosquitoes after immunization with Pfs25-B. This transmission-blocking activity per-

sisted for nearly 4 mo in one monkey and >6 mo in the other (Table 2 B).

Conformational integrity of Pfs25 has been shown to be extremely important for recognition by mAbs that block transmission. Furthermore, linear or improperly folded epitopes of Pfs25 have not been used successfully to elicit transmission-blocking immunity. Expression of the related six-cysteine-containing hEGF in this yeast system resulted in a correctly folded and disulfide-linked polypeptide (14). It was, therefore, somewhat surprising that the conformation-dependent neutralizing mAbs 1C7, 1D2, and 1D3, which are known to react with the same epitope of Pfs25, did not react as strongly with the secreted recombinant Pfs25-B as they did with native Pfs25 on nonreducing SDS-PAGE immunoblots (Fig. 3 C). As the epitopes recognized by these mAbs are destroyed by reduction, but not by SDS, high or low salt concentrations, or extremes of pH (I. A. Quakyi and D. C. Kaslow, unpublished observations), reactivity of mAbs 1C7 and 1D2 by immunoblot with recombinant Pfs25 is a sensitive assay for proper disulfide bond formation at or about the mAb epitope. mAb 1C7 showed clear reactivity with Pfs25-B under nonreducing conditions (Fig. 3 B). In quantitative studies, however, mAbs 1D2 and 1C7 were found to show markedly reduced reactivities with Pfs25-B when compared with native Pfs25. For example, we could demonstrate extensive reactivity of mAb 1D2 with native Pfs25, which was isolated from 5×10^6 parasites, under conditions where no reactivity could be observed with $10 \mu\text{g}$ of Pfs25-B (Fig. 3 C). A typical yield of native Pfs25 has been shown to correspond to $\sim 10 \mu\text{g}/10^9$ parasites (10). This very weak reactivity of mAb 1D2 with secreted Pfs25-B indicates that this particular transmission-blocking epitope is not completely recreated in the recombinant protein. Removal of potential N-linked glycosylation sites, or lack of a glycosylphosphatidylinositol anchor, could explain these findings. A more likely explanation, however, is improper disulfide bond formation. The disulfide bond structure of the secreted Pfs25-B was examined using two other immunological reagents. P7, an antiserum made to a mixture of synthetic peptides from the fourth EGF-like domain of Pfs25, does not recognize native Pfs25. P7 only recognizes fully reduced Pfs25, and thus serves as

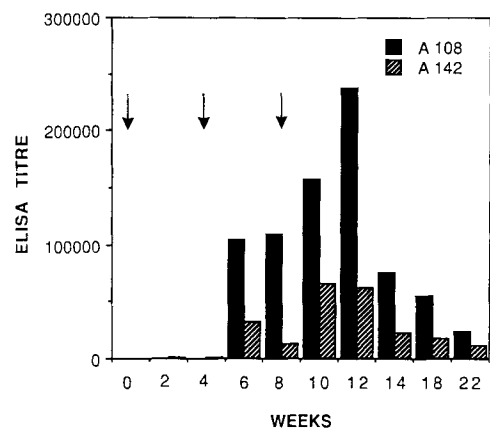


Figure 2. Antibody titers of *Aotus* monkeys immunized with Pfs25-B and MTP-MF59 adjuvant. Monkeys were injected intramuscularly with three $100\text{-}\mu\text{g}$ doses of Pfs25-B in MF59 (7) at four weekly intervals (\downarrow). Titers were measured by ELISA every 2 wk, and sera taken at weeks 0, 12, and 22 were used for mosquito feeding experiments (Table 2 B).

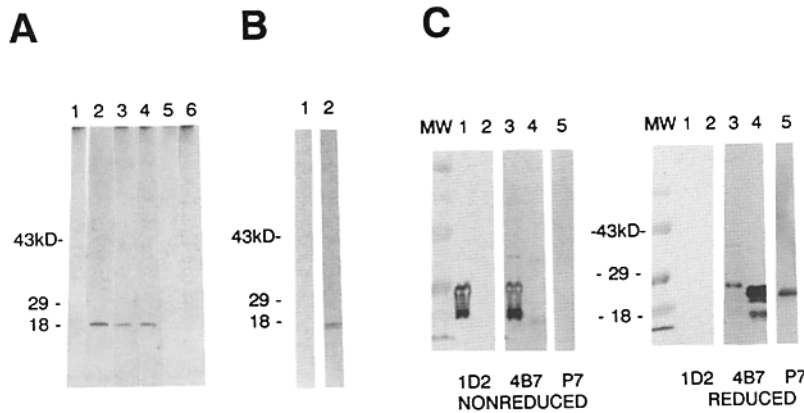


Figure 3. Immunoblot analysis of naive and recombinant Pfs25. (A) Native Pfs25 (Triton X-100 extract from 10^6 5-h zygotes per lane) were fractionated by 4–20% SDS-PAGE under nonreducing conditions, and electroblotted to nitrocellulose (2). Reactivity of Pfs25 with pooled *Aotus* monkey prebleed sera (lane 1), mAb 1D2 (lane 2), immunized monkeys A142 and A108 (lanes 3 and 4), and control monkeys A11 and 1129 (lanes 5 and 6). (B) Reactivity of recombinant Pfs25-B with a control mAb against Pgs25, the *P. gallinaceum* homologue of Pfs25 (lane 1), and with mAb 1C7 (lane 2). (C) Pfs25-B (10 µg per lane; lanes 2, 4, and 5) and native Pfs25 (Triton X-100 extract from 5×10^6 5-h zygotes per lane; lanes 1 and 3) were fractionated similarly, in the presence (reduced) or absence (nonreduced) of 5% 2-ME and electroblotted to nitrocellulose (2). Binding of a 1:200 dilution of mAb 1D2 (lanes 1 and 2), mAb 4B7 (lanes 3 and 4), or antisera P7 (lane 5). In all cases, electroblotted proteins were detected by alkaline phosphatase-labeled goat anti-mouse sera (Pico-Blue; Stratagene). MW, prestained protein markers in kilodaltons (Bethesda Research Laboratories).

an assay for the complete absence of disulfide bonds (I. A. Quakyi et al., manuscript in preparation). P7 reacts with Pfs25-B on reducing but not on nonreducing SDS-PAGE immunoblots, suggesting that Pfs25-B contains some disulfide bonds in the fourth EGF-like domain. mAb 4B7, a previously undescribed mAb to Pfs25 that reacts quite strongly with nonreduced Pfs25 and weakly with reduced Pfs25, clearly reacts with nonreduced Pfs25-B. The reactivity increased markedly upon reduction of Pfs25-B. These data suggest that the mAb 4B7 epitope is improperly formed in Pfs25-B, and further that the decreased reactivity with Pfs25-B, as compared with parasite-produced Pfs25, is not a result of post-translational modification. Thus, although Pfs25-B appears to possess only partial conformational integrity, the yeast-derived product was still fully capable of inducing transmission-blocking immunity in both mice and monkeys.

It has been proposed that the homology of Pfs25 to EGF might give rise to an autoimmune response in individuals immunized with Pfs25. Our data indicate that this is not likely to be the case. First, transmission-blocking antisera did not recognize recombinant hEGF in ELISA (Table 1), nor did antisera to hEGF recognize Pfs25-B in such an assay (Table

1). Second, antisera generated against Pfs25-B did not block EGF activity in either cell proliferation (14) or receptor-binding (15) assay systems (data not shown). We anticipate that cross-reactivity of antibodies to Pfs25 with hEGF, as well as other members of the polypeptide family that contain EGF-like motifs, may not be a complication of this vaccination strategy.

Several recombinant DNA-derived antigens or synthetic peptides of malaria parasites have been shown to be only weakly immunogenic in humans (16, 17). Since Pfs25 is not expressed at the gametocyte stage, but appears only during sexual development in the mosquito, and has, therefore, not been subjected to immune pressure in the human host, it might be anticipated that the immunogenicity of Pfs25-B in humans will closely parallel that observed in experimental animals. Furthermore, transmission-blocking immunity elicited in both mice and monkeys was observed with MTP-MF59, an adjuvant that has been shown previously to be safe and efficacious in humans (13). These studies suggest that recombinant DNA-derived Pfs25 is a promising candidate for the study of transmission-blocking immunity against *P. falciparum* in humans.

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Table 2. Inhibition of Oocyst Development by Sera from Immunized Animals

	Sample	Mean oocyst numbers (range)	Infectivity percent of control	Mosq. infected/ mosq. dissected
A.	Pooled (preimmune)	7.6 (1-22)	100%	16/16
	Pfs25-B (CFA/IFA)	0 (0-1)	<1%	1/19
	Pfs25-B (MF59)	0 (0)	0%	0/18
	Falc 2.3 (MF59)	4.2 (0-13)	55%	17/18
	mAb 1D3	0 (0)	0%	0/16
B.	A108 (preimmune)	39 (0-89)	100%	20/21
	A142 (preimmune)	25 (5-75)	100%	21/21
	A108 (wk 12)	0 (0-1)	<1%	1/24
	A142 (wk 12)	0 (0)	0%	0/20
	A108 (wk 22)	0 (0)	0%	0/28
	A142 (wk 22)	2.6 (0-35)	10%	15/26
	mAb 1D2	0 (0-1)	<1%	1/20
	mAb 4B7	0 (0)	0%	0/24

(A) Sera from mice immunized with Pfs25-B or falc 2.3 (Table 1) were assayed for transmission-blocking activity and compared with the mAb 1D3. Mosquitoes were fed on either the mouse sera or mAb ascites fluid at a 1:2 dilution of the feed (2, 3). Mosquitoes were dissected after 7-8 d, and the numbers of infected mosquitoes and oocysts determined. Infectivity was scored as the number of oocysts found in the sample divided by the number found in the pooled prebled control ($\times 100\%$). (B) The ability of sera from two immunized *Aotus* monkeys to block oocyst development was measured as above. Here, prebled sera from each monkey (numbers A108 and A142) were used as controls. Transmission-blocking activities were measured at 12 and 22 wk. In this experiment, mAbs 1D2 and 4B7 were also shown to block oocyst appearance at a 1:2 dilution, whereas sera from two monkeys that were injected with MF59 alone failed to inhibit oocyst development at any of the bleeds assayed (not shown).

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