

sequence throughout the genome) and a number of them encode repeated stretches of amino acid sequences (4), and these features have been proposed as the major factors limiting *P. falciparum* protein expression in conventional cell-based systems (5–7). Moreover, the presence of glycosylation machinery in eukaryotic cell-based protein expression systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (8–10). There are also constraints such as requirement of disulfide bond formation if the target protein requires it for its bioactivity and requirement of preparation of large quantities of antigen for immunization. The above limiting factors are impediments completely insurmountable in the case of eukaryotic cell-based expression systems such as yeast, baculovirus, or Chinese hamster ovary cell. But fortunately we found that the wheat germ cell-free system can surmount most of the above impediments in the way of finding and developing malaria vaccine candidates (11).

In this chapter, we describe how the wheat germ cell-free system is effective (1) in producing properly folded good quality protein (2) in sufficient quantities (3) that too without the need for codon optimization, using the leading malaria vaccine candidate Pfs25 as an example. And we also describe that (1) proteins produced by the wheat germ cell-free system can be easily purified using simple affinity chromatography, (2) they can be directly used for immunization, and (3) the antibody raised against the proteins are functional in our biochemical, immunocytochemical, and biological analyses. Therefore we hope the wheat germ cell-free system may have dramatic impact on malaria vaccine research.

2. Materials

2.1. Parasite cDNA Preparation, PCR Amplification, and Construction of the DNA Template for Transcription

1. Malaria parasite pellet, *P. falciparum* 3D7 strain (available from Malaria Research and Reference Reagent Resource Center managed by ATCC, Manassas, VA, see Note 1) stored at -80°C in the presence of Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland).
2. RNeasy Micro Kit (QIAGEN, Valencia, CA).
3. SuperScript III RT First-Strand Synthesis System (Invitrogen, Carlsbad, CA).
4. Oligonucleotide primers (see Note 2). Design the 5' primers for the target to be amplified as follows: desired restriction site followed by a 30-mer of unique sequence covering the 5' region of the open reading frame containing the start codon.

Design the 3' primers, desired restriction site followed by a 30-mer of unique sequences covering the 3' region of the open reading frame upstream of the termination codon. Either 5' or 3' primer may contain nucleotide sequence coding hexa-histidine tag for Nickel affinity purification.

5. Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA).
6. Thermal cycler (MJ Research, Waltham, MA) (see Note 3).
7. Plasmid of Ehime University (pEU)-E01 protein expression vector specialized for the wheat germ cell-free system.
8. Ligation high ligation reagent (Toyobo, Osaka, Japan).

2.2. Antigen Scale Cell-Free Protein Synthesis and Affinity Purification

1. pEU-E01 plasmid that contains target cDNA.
2. 5× transcription buffer (TB): 400 mM HEPES-KOH (pH 7.8), 80 mM magnesium acetate, 10 mM spermidine, and 50 mM DTT.
3. Nucleotide tri-phosphates (NTPs) mix: a solution containing 25 mM each of ATP, GTP, CTP, and UTP.
4. SP6 RNA polymerase and RNasin (80 U/mL, Promega, Madison, WI).
5. 40 mg/mL creatine kinase.
6. 240 OD/mL WEPRO®1240H (CellFreeSciences, Matsuyama, Japan).
7. 1× Translational substrate buffer (SUB-AMIX): 30 mM HEPES-KOH (pH 7.8), 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 4.0 mM DTT, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate.
8. 6-well microplate (Greiner, Frickenhausen, Germany).
9. Nickel-nitrilotriacetic acid agarose beads (Qiagen).
10. Poly-Prep chromatography column (Bio-Rad, Hercules, CA, USA).
11. Phosphate buffered saline (PBS): Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄ (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by diluting one part with nine parts of water.
12. 1× Wash buffer: 1× PBS supplemented with 30 mM of imidazole and 300 mM NaCl.
13. 1× Elution buffer: 1× PBS supplemented with 500 mM of imidazole and 300 mM NaCl.

**2.3. Characterization
of the Target
Molecules by Confocal
Immunofluorescence
Microscopy**

1. Female BALB/c mice 6–8 weeks of age (Kitayama Labes, Ina, Japan).
2. Freund complete and incomplete adjuvant (Wako Pure Chemical, Osaka, Japan)
3. CF11 cellulose powder (Whatman, Maidstone, UK)
4. 50% Percoll: Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ) diluted 1:1 (v/v) with 2× PBS
5. Ookinete culture medium: RPMI1640 medium (Invitrogen) supplemented with 50 µg of hypoxanthine per mL, 25 mM HEPES, 20% heat-inactivated fetal calf serum (Invitrogen), 24 mM NaHCO₃, %U of penicillin per mL, and 5 µg of streptomycin per mL (pH 8.4).
6. Eight-well Multitest slides (Flow Laboratories, McLean, VA).
7. Blocking and dilution buffer: 5% (w/v) nonfat dry milk in PBS prepared freshly.
8. Secondary antibody: Antimouse IgG conjugated to Alexa488 (Invitrogen) (see Note 4).
9. Nuclear stain: 2 mg/mL 1,000× DAPI (4,6-diamidino-2-phenylindole) stock solution in 100% methanol.
10. Mounting medium: ProLong Gold Antifade Reagent (Invitrogen) (see Note 5).

**2.4. Malaria
Transmission-
Blocking Vaccine
Efficacy Assay**

1. Malaria parasite-infected blood specimen collected from malaria patient, under informed consent, at the clinics in Thailand.
2. *Anopheles dirus* mosquitoes reared in the insectary, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand (see Note 6).
3. Anti-Pfs25 sera produced in BALB/c mice.
4. Malaria naïve human AB-type serum obtained from volunteers lived in Bangkok.
5. Custom made water-jacketed membrane-feeding apparatus.
6. Parafilm M (Alcan Packaging, Neenah, WI)

3. Methods

One of the well-known difficulties in malaria research is the PCR amplification of the target sequence because nucleotide sequences of *P. falciparum* genes are highly A/T rich. This feature requires longer primers than those for the G/C rich organism to amplify

the target sequences by PCR. Moreover, there are a number of malaria proteins that contain stretches of amino acid repeat motifs and also a number of malaria genes that contain multiple adenine-rich nucleotide sequences (A-islands) in the coding region. This makes the cDNA cloning dramatically difficult, because these A-islands sometimes cause different number of A-nucleotides in each plasmid clone that causes frameshift. Therefore, the initial hurdle for the malaria protein production is the cDNA-cloning; these factors also require consideration on the plasmid construct design. The following methods explain (1) PCR amplification and cloning of the parasite ORF into the pEU expression vector, (2) synthesis of mRNA using expression vector, (3) translation of prepared mRNA in bilayer system, and (4) biological qualification of the synthesized proteins, which includes the recognition of the parasite molecule with the antibody raised against the recombinant malaria protein. This can be accomplished through indirect immunofluorescence microscopy and vaccine efficacy assay.

3.1. Parasite cDNA Preparation and PCR amplification of DNA-Template Construction for Transcription

1. Extract total RNA from cultured *P. falciparum* 3D7 parasites, then reverse transcribe the mRNA into cDNA by using SuperScript III RT First-Strand Synthesis System.
2. PCR amplify the desired target using Phusion™ High-Fidelity DNA Polymerase.
3. Clone the PCR products (see Fig. 8.1) into the pEU-E01 plasmid using Ligation high ligation reagent.
4. Confirm the sequence of the cloned insert (see Note 7).
5. Purify the plasmid.

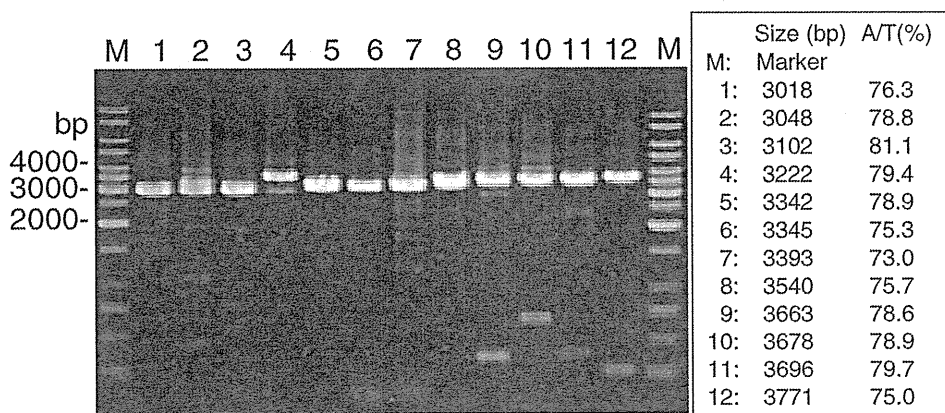


Fig. 8.1. Efficient PCR amplification of the *Plasmodium falciparum* cDNAs. Twelve cDNA targets were selected from *P. falciparum* 3D7, randomly. The cDNA targets were PCR amplified using Phusion™ High-Fidelity DNA Polymerase, under the following conditions: 98°C for 2 min and then 40 cycles at 98°C for 15 s, 55°C for 20 s, and 68°C for 2 min, followed by a final extension at 68°C for 10 min. The amplified products were visualized in a 0.8% agarose gel stained with ethidium bromide. All of the targets ranging from 3,018 bp (lane 1) to 3,771 bp (lane 12) were successfully amplified even in the presence of very high A/T contents (i.e., 73.0–81.1%).

3.2. Antigen Scale Cell-Free Protein Syntheses of Malaria Protein and Affinity Purification

An example of the methods and the results for Pfs25 is described.

1. Prepare a plasmid clone which has an insert as shown in Fig. 8.2A.
2. Incubate 200 μ L of transcription mixture containing 20 μ g of the plasmid DNA, 1 \times TB, 2.5 mM each of NTPs, 200 U of SP6 RNA polymerase, and 200 U of RNasin for 6 h at 37°C.

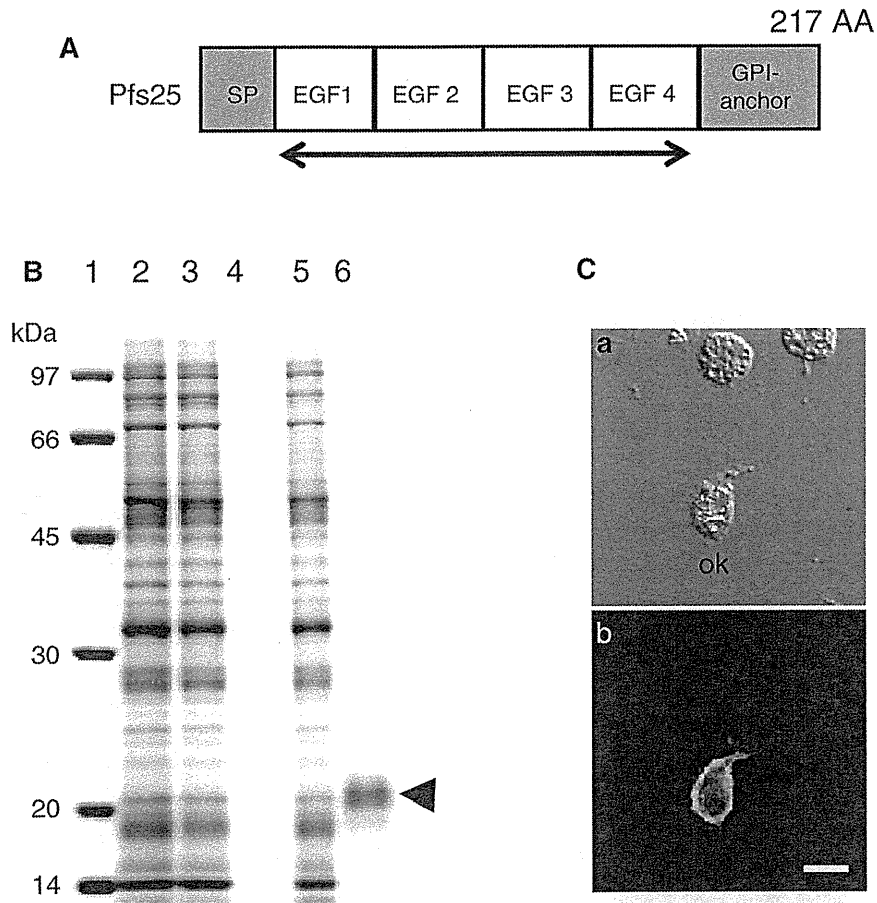


Fig. 8.2. Cloning, expression, and immunolocalization of Pfs25, a leading malaria transmission-blocking vaccine candidate. **(A)** Schematic representation of Pfs25. Pfs25 was expressed without the nucleotide sequences coding for the signal peptide (SP) and the GPI anchor (*arrow*). This Pfs25 gene was amplified by PCR from the *P. falciparum* 3D7 strain using antisense primer that contains nucleotide sequence encoding for hexa-histidine tag at the C terminus and subcloned into pEU-E01 plasmid at the EcoRV site. The A/T content of the *pfs25* insert was 70%. **(B)** The expression, affinity purification, and SDS-PAGE of recombinant Pfs25. Recombinant Pfs25 was expressed using the wheat germ cell-free method, affinity purified by Nickel affinity chromatography, and size-fractionated by 12.5% SDS-PAGE under reducing condition and stained with CBB. *Lane 1*, molecular mass markers in kDa; *lane 2*, total translation mixture; *lane 3*, supernatant fraction of the translation mixture; *lane 4*, pellet fraction of the translation mixture; *lane 5*, flow-through fraction after the affinity purification, *lane 6*; affinity-purified recombinant protein (*arrow head*). **(C)** The immune serum raised against recombinant Pfs25 specifically recognized native Pfs25 proteins expressed on the surface of *P. falciparum* immature ookinetes (ok). **(a)** Differential interference contrast and **(b)** fluorescence confocal images were obtained on a LSM5 PASCAL microscopy (Carl Zeiss MicroImaging, Thornwood, NY) using a 63 \times oil-immersion lens. *Bar*= 5 μ m.

3. Mix the transcription solution containing transcribed mRNA (see Subheading 3.2, step 1) with 200 μ L of WEPRO®1240H (240 OD/mL) supplemented with 0.4 μ L of creatine kinase (40 mg/mL). Add 4.4-mL of 1 \times SUB-AMIX into a single well of a 6-well plate and then underlay the above transcription mixture and incubate at 17°C for 16 h.
4. Add imidazole (pH 8.0) in the translation reaction mixture (final concentration, 20 mM) and then add 80 μ L of 50% slurry of Ni-NTA beads.
5. Incubate the tube for 16 h on a continuous rotator, at 4°C, for the binding of proteins on to the beads.
6. Transfer the solution with the beads into a Poly-Prep column.
7. Wash the beads by 0.4 mL of wash buffer three times and then elute the recombinant protein with 80 μ L of elution buffer five times.
8. Analyze the purified protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition, and the bands were visualized with Coomassie brilliant blue (see Fig. 8.2B).

3.3. Preparation of Antiserum and Parasite Antigen for Indirect Immunofluorescence Assay

3.3.1. Antiserum Preparation

1. Immunize female BALB/c mice (6–8 weeks of age) by subcutaneous injection with affinity purified recombinant proteins emulsified with the same volume of Freund's complete adjuvant as a priming dose and then administer two additional booster doses with Freund's incomplete adjuvant at 3-weeks interval.
2. Collect blood by cardiac puncture a week after the final boost under anesthesia and then separate sera after the coagulation of the blood (see Note 8).

3.3.2. Preparation of Parasite Antigens from the Cultured Ookinetes of *Plasmodium falciparum*

1. Collect peripheral blood with heparinized syringes from malaria patients under written informed consent. Purify gametocytes by passing 5–10 mL of blood through CF-11 column to remove leukocytes followed by 50% Percoll density gradient centrifugation (350 $\times g$ for 25 min at room temperature).
2. Collect the interface rich in gametocytes on the Percoll cushion into a new tube. Wash them twice with PBS and then culture this parasite pellet to ookinete in 1 mL of ookinete medium for 24 h at 24°C in air.
3. Wash the cultured parasite preparations rich in ookinetes twice with PBS and then spot them on 8-well Multitest slides and fix them with ice-cold acetone for 5 min.
4. Store the slides at –80°C until use.

3.3.3. Staining Procedure for Immunofluorescence Assay

1. Take the desired number of antigen slides out from the freezer and then place them quickly in a desiccator until they are brought to room temperature.
2. Block the slides with blocking buffer for 30 min at 37°C in the humidified chamber.
3. Incubate with anti-Pfs25 immune sera (1:100 dilution with blocking buffer) for 1 h at 37°C (see Note 9).
4. Wash the slides with ice-cold PBS for 5 min and incubate with secondary antibody (1:500) and DAPI (1:1,000) diluted with blocking buffer for 30 min at 37°C, followed by washing with ice-cold PBS for 5 min. Mount the slides with Prolong Gold Antifade Reagent and incubate the slides for overnight at room temperature to allow complete solidification of the mounting medium.
5. View the slides under confocal microscopy. Excitation at 488 nm induces the Alexa Fluor 488 fluorescence (green emission) for the Pfs25 (Fig. 8.2Cb), while the differential interference contrast image (Fig. 8.2Ca) is also captured. For example, the fluorescent signal for Pfs25 on the surface of ookinete is shown in Fig. 8.2Cb.

3.4. Malaria Transmission-Blocking Vaccine Efficacy Assay

1. Collect peripheral blood into heparinized syringe from a volunteer patient.
2. Aliquot the collected blood into tubes (300 µL/tube) and remove plasma by a brief centrifugation.
3. Dilute mouse immune sera into 1:2, 1:8, and 1:32 (v/v) with heat-inactivated normal human AB serum prepared from Thai malaria naïve donors.
4. Mix each diluted test serum with *P. falciparum*-infected blood cells (1:1, v/v) and incubate for 15 min at room temperature.
5. Place the mixture into a water-jacketed membrane feeding apparatus (see Fig. 8.3a) whose bottom is sealed by Parafilm M kept at 37°C with circulating water outside of the feeder to allow starved *A. dirus* mosquitoes (Armed Forces Research Institute of Medical Sciences) to feed on the blood meals for 30 min (see Fig. 8.3b).
6. Remove unfed mosquitoes by manual aspiration and maintain only fully engorged mosquitoes for a week by giving 10% sucrose water in the insectary.
7. Dissect 20 mosquitoes for each mouse test immune serum and count the number of oocysts developed within the mosquito midgut under the microscope by staining with 0.5% mercurochrome. The transmission-blocking vaccine efficacy is assessed by the number of oocysts per mosquito (see Fig. 8.3c).

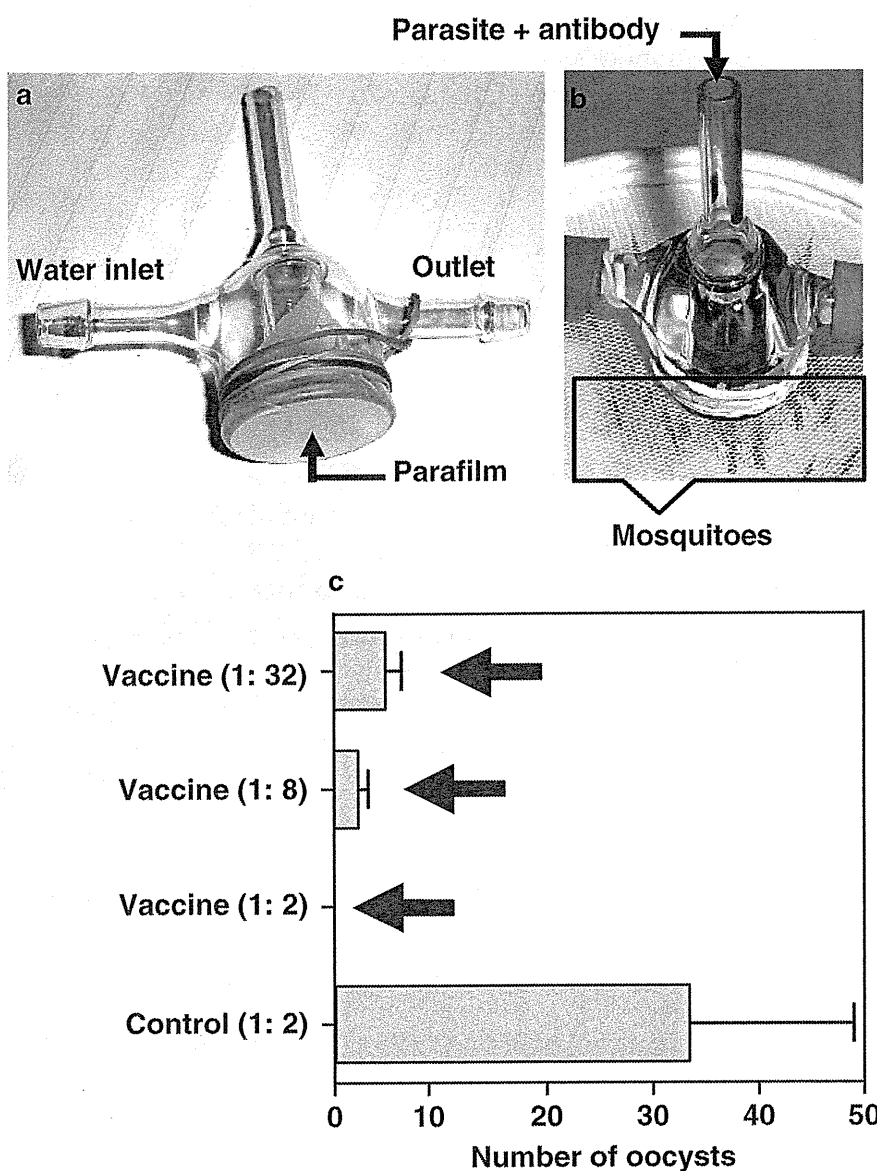


Fig. 8.3. Transmission-blocking efficacy of antibodies against *Plasmodium falciparum* parasites. (a) A membrane feeding apparatus with its bottom sealed by Parafilm M. (b) The apparatus is kept at 37°C with circulating water outside of the feeder to allow mosquitoes to feed on the infected blood mixed with test and control sera. (c) The median numbers of oocysts per mosquito ($n=20$) (bars) with interquartile ranges (lines on top of the bars) were compared among groups of mosquitoes fed on either anti-Pfs25 serum serially diluted or control mouse serum. Dilution range of test immune serum used is shown as 1:2 to 1:32. Arrows indicate statistically significant differences compared to the control group analyzed using Kruskal–Wallis test ($P<0.05$).

4. Notes

1. The Malaria Research and Reference Reagent Resource Center (MR4) is a central source of quality reagents to the malaria research community. Materials available to registered users include parasites, mosquito vectors, antibodies, antigens, gene

libraries, etc. MR4 Web site (<http://www.mr4.org/Home/tabid/93/Default.aspx>) also provides a lot of useful information such as online protocol book named *Methods In Malaria Research* (version 5.2). This book includes protocols that are useful to malaria research, for example, parasite culture methods, immunological assays, or molecular biological techniques.

2. For the efficient PCR amplification, the melting temperature of oligonucleotide primer should be higher than 70°C and it can be roughly calculated by the following formula; $2^{\circ}\text{C} \times (\text{A} + \text{T}) + 4^{\circ}\text{C} \times (\text{G} + \text{C})$. This is one of the essential factors for the efficient PCR amplification of *P. falciparum* because the *P. falciparum* genome is A/T rich.
3. Thermal cycler, which has gradient temperature function, is useful when optimizing the PCR condition, such as testing a lot of different annealing temperatures.
4. For the multiple labeling experiment, check the quality of the secondary antibodies in advance. For example, some of the commercially available secondary antibodies recognizing mouse IgG are highly cross-adsorbed using bovine, goat, human, rabbit, and rat IgG and also human serum. This secondary antibody can be useful for the double labeling with both mouse and rat primary antibodies.
5. This antifade reagent will solidify, and the sample can be saved for months after mounting, and it also offers enhanced resistance to photobleaching. This criterion is very important for the high-quality imaging because this reagent allows the repeated scanning with the confocal laser microscope until the satisfactory images are obtained.
6. *A. dirus* is a major malaria vector mosquito species in Thailand. This mosquito line is established and adapted as one of the laboratory lines.
7. Pay special attention to confirm the number of adenine nucleotides in the A-islands present in the target region. Otherwise wrong number of adenine nucleotides cause frameshift. If a plasmid clone contains wrong number of adenine nucleotides, verify another plasmid clone which sometimes might have a correct number of adenine nucleotides in the A-island.
8. The antisera for immunofluorescence assay can be saved by addition of sodium azide (final concentration, 0.02%) (Caution highly toxic) and stored at 4°C to prevent repeated freeze thaw cycles which damage the antibodies. However, the antisera for the biological assay such as membrane feeding should not contain any preservatives. In this case, all the antisera are kept frozen at -80°C.

9. One well in the 8-well Multitest slide can hold up to 20 μ L of the blocking or antibody solution. In the case of 24-well Multitest slide, one well can hold up to 10 μ L of the solution.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research 18390129 and 19406009; Scientific Research on Priority Areas 21022034 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and, in part, by a Grant-in-Aid of the Ministry of Health, Labour, and Welfare (H21-Chikyukibo-ippan-005), Japan.

References

1. Greenwood, B. and Mutabingwa T. (2002) Malaria in 2002. *Nature* **415**, 670-672.
2. Greenwood B. (2009) Can malaria be eliminated? *Trans. R. Soc. Trop. Med. Hyg.* **103S**, S2-S5.
3. Richie, T. L. and Saul, A. (2002) Progress and challenges for malaria vaccines. *Nature* **415**, 694-701.
4. Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W. et al., (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**, 498-511.
5. Aguiar, J. C., LaBaer, J., Blair P. L., Shamilova, V. Y., Koundinya, M., Russell, J. A., et al. (2004) High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* **14**, 2076-2082.
6. Mehlin, C., Boni, E., Buckner, F. S., Engel, L., Feist, T., Gelb M. H., et al. (2006) Heterologous expression of proteins from *Plasmodium falciparum*: results from 1000 genes. *Mol. Biochem. Parasitol.* **148**, 144-160.
7. Vedadi, M., Lew, J., Artz, J., Amani M., Zhao, Y., Dong, A., et al. (2007) Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. *Mol. Biochem. Parasitol.* **151**, 100-110.
8. Gowda, D. C., and Davidson, E. A. (1999) Protein glycosylation in the malaria parasite. *Parasitol. Today* **15**, 147-152.
9. Kedees, M. H., Azzouz, N., Gerold, P., Shams-Eldin, H., Iqbal, J., Eckert, V., et al. (2002) *Plasmodium falciparum*: glycosylation status of *Plasmodium falciparum* circumsporozoite protein expressed in the baculovirus system. *Exp. Parasitol.* **101**, 64-68.
10. Samuelson, J., Banerjee, S., Magnelli, P., Cui, J., Kelleher, D. J., Gilmore, R., et al. (2005) The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. *Proc. Natl. Acad. Sci. USA* **102**, 1548-1553.
11. Tsuboi, T., Takeo, S., Iriko, H., Jin, L., Tsuchimochi, M., Matsuda, S., et al. (2008) Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect. Immun.* **76**, 1702-1708.



The wheat germ cell-free protein synthesis system: A key tool for novel malaria vaccine candidate discovery

Takafumi Tsuboi^{a,b,*}, Satoru Takeo^a, Thangavelu U. Arumugam^a, Hitoshi Otsuki^c, Motomi Torii^c

^a Cell-free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan

^b Venture Business Laboratory, Ehime University, Matsuyama, Ehime 790-8577, Japan

^c Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Toon, Ehime 791-0295, Japan

ARTICLE INFO

Article history:

Received 24 August 2009

Received in revised form 13 October 2009

Accepted 28 October 2009

Available online 11 November 2009

Keywords:

Malaria

Plasmodium falciparum

Post-genome

Vaccine candidate discovery

Wheat germ cell-free protein synthesis

ABSTRACT

Malaria kills more than a million people a year, causes malady in about three hundred million people and poses risk to approximately 40% of the world's population living in malarious countries. This disease is re-emerging mainly due to the development of drug-resistant parasites and insecticide-resistant mosquitoes. Therefore, we are now forced to resort to remedy through vaccination. Until now, not even a single licensed malaria vaccine has been developed despite intensive efforts. Even the efficacy of RTS,S, the most advanced and promising vaccine candidate in the pipeline of malaria vaccine development, was only around 50% based on a number of clinical trials. These facts urge malaria researchers to urgently enrich this pipeline, as much as possible, with potential vaccine candidates. With the availability of malaria genome database, the enrichment of this pipeline is possible if we could now employ an efficient protein expression technology to decode the malaria genomic data, without any codon optimization, into quality recombinant proteins. Then, these synthesized recombinant proteins can be characterized and screened for discovering novel potential vaccine targets. The wheat germ cell-free protein synthesis system will be a promising tool to this end. This review highlights the recent successes in synthesizing quality malaria proteins using this tool.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Malaria kills more than a million people a year, causes malady in about three hundred million people and poses risk to around 3.3 billion people (WHO, 2008). Despite the parasite's complex life cycle, high level of antigenic diversity and mechanism of immune evasion, naturally acquired immunity to malaria does develop after repeated exposure over a period of several years and this immunity confers protection, against symptomatic disease, high-density parasitemia and death (Doolan et al., 2009; Genton, 2008; Gupta et al., 1999; Marsh and Kinyanjui, 2006), through protective antibodies (Cohen et al., 1961; McGregor, 1964). In addition to the naturally acquired immunities, sterile, long-lasting protective immunity has been convincingly proved in many studies, such as vaccination with radiation-attenuated sporozoites (Clyde, 1975; Hoffman et al., 2002; Nussenzweig et al., 1967), and inoculation of infective sporozoites to human volunteers under a prophylactic regimen of chloroquine (Roestenberg et al.,

2009). These facts strongly support the reasoning of incorporating malaria vaccines as one of the components of malaria control measures.

2. Malaria vaccine development

The efficacy of malaria control through current interventions that employs drugs and insecticides may not be sustained too long since they rely on too few compounds (Genton, 2008). In fact, the disease is re-emerging mainly due to the emergence of drug-resistant parasites (Greenwood and Mutabingwa, 2002). Very recently, parasites have developed resistance even to the hitherto-promising artemisinin (Dondorp et al., 2009). Therefore, we have to develop and employ malaria vaccines as one of the essential components towards the malaria eradication (Greenwood, 2009). The fact that, in spite of intensive efforts, not even a single licensed malaria vaccine has been developed, urges malaria research community to employ efficient post-genomic approaches (Richie and Saul, 2002). Malaria vaccines are generally divided into three groups based on stages of the parasite life cycle targeted by the vaccine. They are pre-erythrocytic vaccines, asexual blood-stage vaccines, and transmission-blocking vaccines. Fig. 1 gives a quick description about vaccine categories, the stages they target, along with malaria vaccine candidates in clinical trials. Detailed descriptions about

* Corresponding author at: Ehime University, Cell-Free Science and Technology Research Center, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan.
Tel.: +81 89 927 8277; fax: +81 89 927 9941.

E-mail address: tsuboi@ccr.ehime-u.ac.jp (T. Tsuboi).

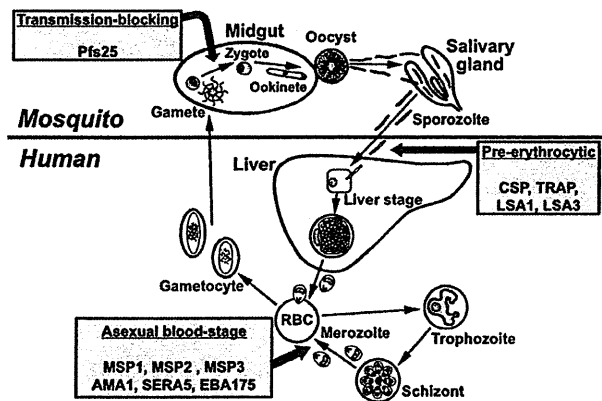


Fig. 1. Malaria vaccines in clinical trials mapped on the parasite life cycle. Malaria vaccines are categorized into three groups based on the target stages of the parasite life cycle. They are pre-erythrocytic, asexual blood-stage, and transmission-blocking vaccines. RBC: red blood cell, CSP: circumsporozoite protein, TRAP: thrombospondin-related adhesion protein, LSA1: liver stage antigen 1, LSA3: liver stage antigen 3, MSP1: merozoite surface protein 1, MSP2: merozoite surface protein 2, MSP3: merozoite surface protein 3, AMA1: Apical membrane antigen 1, SERA5: Serine repeat antigen 5, and EBA175: erythrocyte binding antigen 175. Detailed descriptions about these vaccines were given in other published reviews (Genton, 2008; Richards and Beeson, 2009).

these vaccines are given in other published reviews (Genton, 2008; Richards and Beeson, 2009).

2.1. Pre-erythrocytic vaccines

Pre-erythrocytic vaccines have been designed to prevent entry of sporozoites into hepatocytes and the development of liver stage parasites (Greenwood et al., 2008; Waters, 2006).

2.2. Asexual blood-stage vaccines

Asexual blood-stage vaccines are designed to reduce merozoite invasion, multiplication and growth in order to protect against clinical symptoms and particularly severe disease. It is widely understood that this vaccine induces antibodies that may have roles in prevention of merozoite invasion, clearance of infected erythrocytes, prevention of adhesion and sequestration of parasitized erythrocytes in the vasculature. It is also possible that, in addition to preventing clinical illness, an effective blood-stage vaccine may also contribute to malaria eradication by reducing the efficiency of the transmission of parasites from human host to mosquito by interrupting the blood-stage life cycle in the human body (Duffy, 2007; Genton, 2008; Moll et al., 2007; Richards and Beeson, 2009).

2.3. Transmission-blocking vaccines

Transmission-blocking vaccines are aimed at interrupting the parasite life cycle in the mosquito blood meal. These vaccines elicit antibodies against antigens that are expressed by the sexual stages of the parasite and, thus, stop their subsequent development in the mosquito midgut (Carter, 2001; Tsuboi et al., 2003). These transmission-blocking vaccines, if used in combination with pre-erythrocytic or asexual blood-stage vaccines, might play a key role in finally breaking the transmission of parasites, leading to eradication of the diseases (Targett and Greenwood, 2008).

It is an accepted view that an effective malaria vaccine need to target several stages of parasite and several components of the different stages of parasite (Lasonder et al., 2002) and it must induce

protective immune responses equivalent to, or better than, those provided by naturally acquired immunity or immunization with attenuated whole parasite (Gardner et al., 2002). In order to accelerate the discovery of such vaccine candidates, it is indispensable now to establish and exploit two things. One is the optimal recombinant protein synthesis system for synthesizing malaria proteins on a whole-proteome scale and other is the efficient post-genomic high-throughput approaches for sifting potential vaccine candidates out from this whole malaria proteome.

3. Post-genome approaches for novel malaria vaccine candidate discovery research

As described in Fig. 1, decades of research in the pre-genomic era have identified only a handful of vaccine candidates. With the recent completion of the genome projects of human malaria parasites, *Plasmodium falciparum* (Gardner et al., 2002), *P. vivax* (Carlton et al., 2008), primate malaria parasite, *P. knowlesi* (Pain et al., 2008), and rodent malaria parasite, *P. yoelii* (Carlton et al., 2002), we are now in the post-genome era. However, to tangibly reap the benefits from these genomic data, it is indispensable to thoroughly analyze these data using at least two post-genomic high-throughput approaches. One is the functional approach (otherwise called as reverse vaccinology (Flower, 2008)), and the other is the immunoscreening approach.

3.1. Functional approach

In the functional approach, initially putative vaccine candidates are selected from the genome database based on either patent or latent functional criteria. Then these putative candidates are expressed *in vitro* using effective and efficient protein synthesis system to obtain quality proteins for further downstream vaccine candidate assessment studies (Hall et al., 2005). For example, the genes that are essential for the parasite's survival revealed by gene knockout studies (Cowman and Crabb, 2006), or the genes with signatures of strong immune selective pressure revealed by polymorphisms and diversity studies (Mu et al., 2007), or the genes involved in host cell invasion, or the genes whose products are localized on the cell surface or in the apical organelles of the sporozoite, merozoite, and ookinete could be putative vaccine candidates.

3.2. Immunoscreening approach

In the immunoscreening approach, initially putative vaccine candidates are selected based on their immuno-reactivity with the protective antibodies that are elicited in humans, after natural or experimental infection or after vaccination with attenuated organisms (Hoffman et al., 2002; VanBuskirk et al., 2009). Here extensive set of quality recombinant malaria proteins are synthesized, in small scale, using protein synthesis system and are screened intensively, using immunoassays such as enzyme-linked immunosorbent assay, protein microarray, with a large number of human serum samples obtained from non-immune and immune individuals (Doolan et al., 2008). Then, these immuno-reactive putative vaccine candidates that correlate with protection will be synthesized, in large scale, for further downstream vaccine research.

4. What is the optimal recombinant protein synthesis system for malaria proteins?

Whatever may be the approach employed for novel vaccine candidate discovery research, we do need to have an effective and

efficient recombinant protein synthesis system not only for the initial synthesis, characterization, and downstream assessment of putative vaccine candidates, but also for the mass production of vaccine antigens for vaccination purpose in the later stage. Therefore, the choice of the recombinant protein synthesis method is the most crucial factor. One of the main obstacles in malaria vaccine discovery research is the lack of an optimal protein expression system that can decode A/T-rich, low-complexity-region-containing (i.e., repeated stretches of amino acid sequences) malaria genes into high quality (i.e., properly folded) recombinant proteins that are amenable for malaria vaccine research (Gardner et al., 2002). These obstacles pose major limitation to express *P. falciparum* genes not only in conventional *E. coli* cell-based systems (Aguilar et al., 2004; Mehlin et al., 2006; Vedadi et al., 2007), but also in eukaryotic cell-based expression systems such as yeast, baculovirus, or Chinese hamster ovary cell (Tsuboi et al., 2008).

Since the rate of peptide growth on ribosomes is 5–10 times slower in eukaryotes than in prokaryotes, this slow rate of peptide growth in eukaryotic protein expression system contributes greatly towards correct conformational folding of multidomain proteins and their solubility (Hartl and Hayer-Hartl, 2002; Netzer and Hartl, 1997). Therefore eukaryotic proteins with multiple domains, such as malaria parasite proteins, when expressed either in *E. coli* cell-free or cell-based system, tend to fold incorrectly, and become insoluble (Netzer and Hartl, 1997). After all, the correct folding and solubility of malaria proteins are indispensable critical factors that directly affect the success in our genome-wide search for potential vaccine antigens. From the correct folding point of view, the eukaryotic-based system is greatly advantageous over *E. coli* based prokaryotic system. The eukaryotic-based systems have been widely used to synthesize eukaryotic multidomain proteins in active forms. However, they all suffer from low throughput and low productivity (Endo and Sawasaki, 2006; Goshima et al., 2008). For example, it is well known that the rabbit reticulocytes lysate system has been employed for the production of quality eukaryotic recombinant proteins. However, the yield of this system is very low, and moreover this system has post-translational modification machinery, i.e., glycosylation (Endo and Sawasaki, 2006). Therefore it is disadvantageous to express malaria genes in rabbit reticulocyte system and other eukaryotic cell-based protein expression systems that possess glycosylation machinery, for these systems can produce inappropriately glycosylated recombinant malaria proteins, resulting in incorrect immune responses (Gowda and Davidson, 1999; Kedees et al., 2002). The fact that both the wheat germ system and the malaria parasite do not have glycosylation machinery (Samuelson et al., 2005) is an additional advantage for expressing malaria proteins without any inadvertent glycosylation in wheat germ system. (Endo and Sawasaki, 2006). Among all eukaryotic protein expression systems, wheat germ cell-free translation system is the most suitable for easy handling and achieving high-throughput, high-solubility, and high-productivity in the synthesis of recombinant proteins (Endo and Sawasaki, 2006; Goshima et al., 2008).

From our experience in expressing around five hundred malaria genes using wheat germ cell-free system, we have found that the wheat germ cell-free system can surmount almost all of the above stumbling blocks in the path of post-genome malaria vaccine candidate discovery and therefore can be used for synthesizing high level of soluble malaria recombinant proteins (an important prerequisite for vaccine candidate assessment and large scale vaccine production), and accelerate malaria vaccine discovery (Tsuboi et al., 2008). Some of the successes that have been published by our collaborators and us have been described in the subsequent sections. Therefore we believe that the wheat germ system is the optimal system for synthesis of malaria proteins.

5. Synthesis of malaria proteins using prokaryotic protein synthesis system

There were several trials to achieve genome-wide expression of *P. falciparum* genes using the conventional *E. coli* based protein synthesis system. Aguiar et al. (2004) using *E. coli* cells, were able to express only 39 out of 292 malaria genes cloned in GST-fusion vector. Mehlin et al. (2006) tried to express 1000 genes encoding relatively small (<450 amino acids) malaria cytosolic proteins, in *E. coli*, for the structural analysis. In that study, only 30% of the genes were expressed and only 6.3% of the proteins were soluble, yielding 0.9–406 mg of protein per liter of culture medium. Vedadi et al. (2007) tried to express 182 malaria genes, for the structural analysis, utilizing genetically engineered *E. coli* strain supplemented with tRNAs that allows reading of high number of A/U codons in malaria mRNA. They achieved marginal improvement in protein solubility, i.e., 38 out of 182 (20.9%) proteins tested were soluble. Using an *E. coli* cell-free rapid expression system, Mu et al. attempted to express 108 polymorphic malaria genes that are considered to be under immune selection pressure to find potential vaccine candidates. Expression of proteins was verified via protein blot using antibodies to the histidine tags incorporated into the C-terminus of the expressed proteins. In their study, they found that, out of 108 genes, 65 genes (i.e., 60.2%) could be expressed (Mu et al., 2007). Recently, Doolan et al. (2008) reported the construction of the *P. falciparum* protein microarrays using *E. coli* cell-free protein synthesis system for high-throughput immunoscreening for vaccine candidate discovery. In their study, they obtained >90% efficiency in their effort to express 250 *P. falciparum* genes using *E. coli* cell-free *in vitro* transcription and translation reactions. And they have attributed their high success rate in this system, at least in part, to the fact that the system is supplemented with rare transfer RNAs to help translate A/T-rich genes. However, in the *E. coli* cell-free system, the rate of protein expression reported by Mu et al. (2007) was lower than the rate reported by Doolan et al. (2008). The above experiments proved that *E. coli* cell-free system is better in productivity and throughput than *E. coli* cell-based system, however, these experiments did not prove that the quality of proteins synthesized in *E. coli* cell-free system is amenable enough for vaccine candidate discovery research.

6. A cursory glance at wheat germ cell-free protein synthesis system

Recently, the wheat germ cell-free protein synthesis system was established for high-throughput synthesis of soluble recombinant proteins (Madin et al., 2000) and is now commercially available as simple protein synthesis kits (CFS Co., Ltd., Matsuyama, Japan). Basically there are two protocols established for practical use in this system (Endo and Sawasaki, 2006). The first one is for small scale, high-throughput or parallel synthesis of proteins. It can be applied for initial testing of gene expression and solubility of proteins. This will facilitate genome-wide biochemical annotation of gene products (Sawasaki et al., 2002). The second protocol is for the large scale production of interested proteins (Endo and Sawasaki, 2006; Kamura et al., 2005; Sawasaki and Endo, 2008). This protocol can produce hundreds of micrograms of protein, and also, offer scope for scale up (Sawasaki and Endo, 2008). These two protocols have already been acknowledged as advantageous for producing high quality eukaryotic proteins for structural and functional genomics studies (Goshima et al., 2008; Vinarov et al., 2004). Taken together, this system is versatile for both small and large scale synthesis of quality eukaryotic proteins.

Table 1The recent successes in the synthesis of biologically active malaria proteins using wheat germ cell-free expression system^a.

Gene ID ^b	Gene name	Species ^c	Results ^d	References
Enzymes				
PFD0830w	Dihydrofolate reductase–thymidylate synthase	<i>Pf</i>	Enzyme activities (DHFR, and TS)	Mudeppa et al. (2007)
PVX.087680	Chitinase	<i>Pv</i>	Enzyme activities	Takeo et al. (2009)
PF10.0363	Pyruvate kinase 2	<i>Pf</i>	Ab (WB, IFA)	Maeda et al. (2009)
Sporozoite antigens				
PFC0210c	Circumsporozoite protein	<i>Pf</i>	Ab (WB, IFA)	Tsuboi et al. (2008)
PFD0215c	P52	<i>Pf</i>	Ab (IFA)	VanBuskirk et al. (2009)
PKH.121770	thrombospondin-related adhesion protein	<i>Pk</i>	T cell	Jiang et al. (2009)
Merozoite antigens				
PF11.0344	Apical membrane antigen 1	<i>Pf</i>	Ab (WB, IFA)	Tsuboi et al. (2008)
PF14.0495	Rhoptry neck protein 2	<i>Pf</i>	Ab (IP, WB, IFA, IEM)	Cao et al. (2009)
PY04764	Erythrocyte binding ligand	<i>Py</i>	Ab (WB, IFA, IEM)	Otsuki et al. (2009)
PKH.072850	Merozoite surface protein 1	<i>Pk</i>	T cell	Jiang et al. (2009)
Ookinete antigen				
PF10.0303	Pfs25	<i>Pf</i>	Ab (WB, IFA, TBA)	Tsuboi et al. (2008)

^a The individual articles may be consulted for further details.^b Detailed information is available at the PlasmoDB website. (<http://plasmodb.org/plasmo/>).^c Plasmodium species, *Pf*: *Plasmodium falciparum*, *Pv*: *P. vivax*, *Py*: *P. yoelii*, and *Pk*: *P. knowlesi*.^d Results obtained by the contribution of recombinant proteins synthesized by the wheat germ cell-free system. Ab, antibody; IP, immunoprecipitation; WB, Western blot, IFA, immunofluorescence microscopy, IEM, immunoelectron microscopy. T cell, *in vitro* T cell studies; TBA, transmission-blocking activity.

7. Synthesis of malaria proteins using eukaryotic wheat germ protein synthesis system

Initially, in order to test the suitability of wheat germ cell-free system for high-throughput synthesis of malaria recombinant proteins, we attempted to express 124 genes encoding asexual blood-stage parasite proteins, selected from malaria genome database, PlasmoDB (<http://plasmodb.org/plasmo/>). Out of 124 genes, 93 of them (i.e., around 75%) yielded soluble protein products. However, the magnitude/extent of solubility among these 93 soluble proteins is on an average 65% and ranges from 26% to 100% (Tsuboi et al., 2008). The average yield of full size recombinant proteins was 1.9 µg per 150 µl of reaction mixture and this amount is sufficient for high-throughput screening of antigens that correlates with protection using hyper-immune serum.

The experimental results published by our collaborators and us (Table 1) confirm that wheat germ cell-free protein synthesis system is very suitable for decoding A/T-rich malaria genes without any codon optimization into biologically active malaria proteins. Firstly, the system was able to synthesize active malaria enzymes that are recalcitrant to expression in other systems, such as *P. falciparum* dihydrofolate reductase–thymidylate synthase (Mudeppa et al., 2007), a chitinase of *P. vivax* (Takeo et al., 2009) and pyruvate kinase type-II isozyme of *P. falciparum* (Maeda et al., 2009). Secondly, the system was able to produce a sufficient amount of good quality malaria proteins, such as repeat-rich circumsporozoite protein (Tsuboi et al., 2008), cysteine-rich P52 (VanBuskirk et al., 2009), thrombospondin-related adhesion protein (Jiang et al., 2009), rhoptry neck protein 2 (Cao et al., 2009), erythrocyte binding ligand (Otsuki et al., 2009), merozoite surface protein 1 (Jiang et al., 2009), apical membrane antigen 1 and highly cysteine-rich Pfs25 (a promising transmission-blocking vaccine candidate) (Tsuboi et al., 2008).

Recently, we attempted to express 567 of *P. falciparum* cDNA clones belonging to sporozoite, merozoite, and gametocyte stages in a high-throughput format by the wheat germ cell-free system. Out of 567 genes, 478 of them (i.e., around 84%) yielded soluble protein products (unpublished). Our biochemical, immunocytochemical, and biological analyses have revealed that the recombinant malaria proteins synthesized by this system are of high quality and therefore amenable for the assessment and discovery of potential vaccine targets (Tsuboi et al., 2008). Thus, we

indeed believe that the wheat germ cell-free protein synthesis system is a key tool for decoding malaria genome for malaria proteome and vaccine research.

8. Conclusion

From a malaria vaccine perspective, wheat germ cell-free system is the most suitable system to date for easily achieving high-throughput, high-solubility, and high-productivity in the whole-proteome-scale synthesis of malaria proteins and construction of microarrays of malaria proteins that will accelerate post-genomic novel malaria vaccine candidate discovery.

Acknowledgments

We thank Kana Kato, Aya Tamai, and Mai Tasaka for their technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research 19406009 (TT), Scientific Research on Priority Areas 21022034 (TT) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; and, in part, by a Grant-in-Aid from the Ministry of Health, Labour, and Welfare (H21-Chikyukibo-ippa-005) (TT), Japan.

References

- Aguiar, J.C., LaBaer, J., Blair, P.L., Shamailova, V.Y., Koundinya, M., Russell, J.A., Huang, F., Mar, W., Anthony, R.M., Witney, A., Caruana, S.R., Brizuela, L., Sacchi Jr., J.B., Hoffman, S.L., Carucci, D.J., 2004. High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* 14, 2076–2082.
- Cao, J., Kaneko, O., Thongkukiatkul, A., Tachibana, M., Otsuki, H., Gao, Q., Tsuboi, T., Torii, M., 2009. Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in *Plasmodium falciparum* merozoites. *Parasitol. Int.* 58, 29–35.
- Carlton, J.M., Angiuoli, S.V., Suh, B.B., Kooij, T.W., Perete, M., Silva, J.C., Ermolaeva, M.D., Allen, J.E., Selengut, J.D., Koo, H.L., Peterson, J.D., Pop, M., Kosack, D.S., Shumway, M.F., Bidwell, S.L., Shallom, S.J., van Aken, S.E., Riedmuller, S.B., Feldblyum, T.V., Cho, J.K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L.M., Florens, L., Yates, J.R., Raine, J.D., Sinden, R.E., Harris, M.A., Cunningham, D.A., Preiser, P.R., Bergman, L.W., Vaidya, A.B., van Lin, L.H., Janse, C.J., Waters, A.P., Smith, H.O., White, O.R., Salzberg, S.L., Venter, J.C., Fraser, C.M., Hoffman, S.L., Gardner, M.J., Carucci, D.J., 2002. Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419, 512–519.
- Carlton, J.M., Adams, J.H., Silva, J.C., Bidwell, S.L., Lorenzi, H., Caler, E., Crabtree, J., Angiuoli, S.V., Merino, E.F., Amedeo, P., Cheng, Q., Coulson, R.M., Crabb, B.S., Del Portillo, H.A., Essien, K., Feldblyum, T.V., Fernandez-Becerra, C., Gilson, P.R., Gueye, A.H., Guo, X., Kang'a, S., Kooij, T.W., Korsinczyk, M., Meyer, E.V., Nene, V., Paulsen, I., White, O., Ralph, S.A., Ren, Q., Sargeant, T.J., Salzberg, S.L., Stoeckert, C.J., Sullivan, S.A., Yamamoto, M.M., Hoffman, S.L., Wortman, J.R.,

- S.H., 2009. Preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design. Proc. Natl. Acad. Sci. U.S.A. 106, 13004–13009.
- Vedadi, M., Lew, J., Artz, J., Amani, M., Zhao, Y., Dong, A., Wasney, G.A., Gao, M., Hills, T., Brox, S., Qiu, W., Sharma, S., Diassiti, A., Alam, Z., Melone, M., Mulichak, A., Wernimont, A., Bray, J., Loppnau, P., Plotnikova, O., Newberry, K., Sundararajan, E., Houston, S., Walker, J., Tempel, W., Bochkarev, A., Kozieradzki, I., Edwards, A., Arrowsmith, C., Roos, D., Kain, K., Hui, R., 2007. Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. Mol. Biochem. Parasitol. 151, 100–110.
- Vinarov, D.A., Lytle, B.L., Peterson, F.C., Tyler, E.M., Volkman, B.F., Markley, J.L., 2004. Cell-free protein production and labeling protocol for NMR-based structural proteomics. Nat. Methods 1, 149–153.
- Waters, A., 2006. Malaria: new vaccines for old? Cell 124, 689–693.
- WHO, 2008. World Malaria Report 2008. WHO Press, Geneva, Switzerland.

