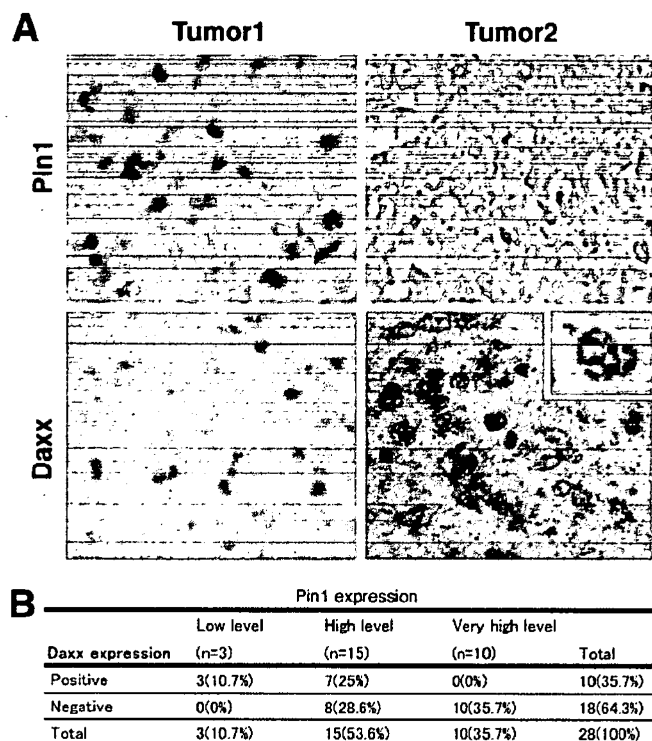

Daxx expression	Pin1 expression Low level (n=3)	High level (n=15)	Very high level (n=10)	Total
Positive	3(10.7%)	7(25%)	0(0%)	10(35.7%)
Negative	0(0%)	8(28.6%)	10(35.7%)	18(64.3%)
Total	3(10.7%)	15(53.6%)	10(35.7%)	28(100%)

FIGURE 8. **Reverse correlation between Pin1 and Daxx expression in human glioblastoma tissues.** *A*, human glioblastoma tissues were stained with anti-Daxx or anti-Pin1 antibody followed by staining with diaminobenzene (DAB; *brown*). *Left*, a representative tumor showing strong staining for Pin1 and negative Daxx staining; *right*, a representative low Pin1-stained glioma with an accumulation of Daxx in the nucleus. Nuclei were stained weakly with hematoxylin. *Inset*, a focal magnification of Daxx staining in nuclear bodies. *B*, a summary of immunohistochemical analysis of a 28-human glioblastoma tissue panel is shown. The levels of Pin1 and Daxx expression were determined, and a significant reverse correlation was confirmed using a Spearman rank test ($p < 0.01$).

cated that Daxx might relocalize from the nucleus to the cytoplasm upon receipt of specific stimuli, including Fas stimulation, oxidative stresses, and glucose deprivation (10, 14). The regulation of Daxx nuclear export appears to be dependent on its phosphorylation on Ser[668], potentially mediated by the HIPK1 kinase (14). This in turn enhances the interaction of Daxx with the nuclear exporter CRM1, which controls its phosphorylation-dependent translocation to the cytoplasm (14). DJ-1 and heat shock protein 27 (HSP27) have been shown to be Daxx suppressor proteins that can directly interact with Daxx and suppress its nuclear export by CRM1, thereby inhibiting ASK1/JNK activation and apoptosis (28, 29). However, our current study has revealed for the first time that in addition to its phosphorylation at the C-terminal Ser[668] residue, Daxx is also phosphorylated on Ser[178] and subsequently targeted by Pin1-mediated prolyl isomerization for degradation via the ubiquitin-proteasome pathway.

The ubiquitin-mediated proteolysis of Daxx has been reported recently. Daxx has been shown to undergo ubiquitination and degradation in response to double strand DNA damage, resulting in the translocation of Ras-association domain family 1C (RASSF1C) to the microtubules (30). Moreover, BTB domain-containing speckle-type POZ protein serves as an adaptor of Daxx that facilitates its ubiquitination by Cul3-based ubiquitin ligase (31). However, these studies did not reveal any details regarding the mechanistic processes underlying the ubiquitination of Daxx following the receipt of proapoptotic stimuli. It is possible that Pin1-mediated prolyl isomerization of Daxx on phosphorylated Ser[178]-Pro, as shown in this study, might affect these ubiquitination processes as they pertain to Daxx.

Our current data also clearly show that both the Pin1 WW domain and PPIase domain are required for the degradation of Daxx, suggesting that the prolyl isomerization of Daxx by Pin1 is important for its polyubiquitination and degradation. Our current findings thus provide the first evidence that Daxx protein stability is regulated by a series of post-translational modifications (*i.e.* phosphorylation and subsequent prolyl isomerization leading to ubiquitination). The future identification of the kinase(s) responsible for phosphorylation of the Ser[178]-Pro Pin1 binding motif of Daxx might further uncover the mechanisms underlying antiapoptotic signaling in tumor cells.

In summary, we demonstrate herein that Pin1 is a negative regulator of Daxx and demonstrate a novel regulatory mechanism of Daxx involving phosphorylation-dependent prolyl isomerization. The targeted inhibition of Pin1 could therefore be a valid therapeutic strategy to induce cellular apoptosis in malignant tumors. This includes gliomas in which aberrantly high Pin1 expression is often observed.

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1 **The Wheat Germ Cell-Free Based Production of Malaria Proteins for Discovery of Novel**
2 **Vaccine Candidates**[†]
3
4 Running title: CELL-FREE MALARIA PROTEIN EXPRESSION
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26 **Abstract.** One of the major bottlenecks in malaria research has been the difficulty in recombinant
27 protein expression. Here, we report the application of the wheat germ cell-free system for the
28 successful production of malaria proteins. For proof-of-principle, the Pfs25, PfCSP and PfAMA1
29 proteins were chosen. These genes contain very high A/T sequences and are also difficult to
30 express as recombinant proteins. In our wheat germ cell-free system, both native and codon
31 optimized version of the Pfs25 genes produced equal amount of proteins. PfCSP and
32 PfAMA1genes without any codon optimization were also expressed. The products were soluble,
33 with yields between 50-200 µg/ml of the translation mixture, indicating that the cell-free system
34 can be used to produce malaria proteins without any prior optimization of their biased codon usage.
35 Biochemical and immunocytochemical analyses of antibodies raised in mice against each protein
36 revealed that every antibody retained its high specificity to the parasite protein in question. The
37 development of parasites in mosquitoes fed patient blood carrying P. falciparum gametocytes and
38 supplemented with our mouse anti-Pfs25 sera was strongly inhibited, indicating that both
39 Pfs25-3D7/WG and Pfs25-TBV/WG retained their immunogenicity. Lastly, we carried out a
40 parallel expression assay of proteins of blood stage P. falciparum. The PCR products of 124 P.
41 falciparum genes chosen from the available database were used directly in a small-scale format of
42 transcription and translation reactions. Autoradiogram testing revealed the production of 93
43 proteins. Application of this new cell-free based protocol for the discovery of malaria vaccine
44 candidates will be discussed.

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45 **INTRODUCTION**

46 Plasmodium falciparum is the protozoan responsible for the widespread return of malaria to
47 tropical countries, particularly in Africa. This re-emergence is generally accredited to two causes;
48 the development of multi-drug-resistant parasites and the development of insecticide-resistant
49 mosquitoes (10). Through decades of work, scientists have learnt that vaccination could be a potent
50 curative, but efforts to develop a successful vaccine have not yet succeeded (25). One of the
51 bottlenecks in vaccine development is at the malaria protein production step, and is mainly due to
52 the lack of a methodology to enable preparation of quality proteins in an efficient manner. P.
53 falciparum genes have a very high A/T content (average 76% per gene) and a number of them
54 encode repeated stretches of amino acid sequences (8), and these features have been proposed as
55 the major factors limiting P. falciparum protein expression in cell-based systems. Moreover, the
56 presence of glycosylation machinery in eukaryotic cell-based systems can produce inappropriately
57 glycosylated recombinant malaria proteins, resulting in incorrect immune responses (9,21,26). In
58 fact, the three pioneering genome-wide studies on the production of P. falciparum proteins in
59 cell-based systems faced serious problems. For instance, Aguiar et al. (1) was able to obtain
60 expression in Escherichia coli cells of only 39 of 292 malaria genes cloned into the GST-fusion
61 vector. Mehlin et al. carried out an even more challenging trial in which 1000 genes encoding
62 relatively small (<450 amino acids) malaria cytosolic proteins were expressed in E. coli (24). In
63 that study only 30% of the genes were expressed and only 6.3% of the proteins were soluble,
64 yielding 0.9 mg to 406 mg of protein per liter of culture medium. The other approach used an
65 engineered E. coli strain with tRNAs genetically supplemented to allow reading of the high number
66 of A/U codons in malaria mRNA (31). A significant improvement in protein solubility, up to
67 20.9%, was observed (38 out of 182 proteins tested were soluble). However, although the E. coli
68 translation system is known to support folding of prokaryotic and small eukaryotic proteins, the

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69 multidomain proteins common in eukaryotes tend to fold incorrectly in the E. coli system, resulting
70 in the formation of inclusion bodies.

71 Through decades of laborious work, scientists have identified three leading vaccine
72 candidates from the pool of P. falciparum proteins: Pfs25 (19), PfCSP (5,12,34), and PfAMA1
73 (6,11). Pfs25, a zygote/ookinete surface protein, is a promising candidate as a
74 transmission-blocking vaccine. This protein is composed of four tandem epidermal growth
75 factor-like (EGF-like) domains, containing three putative N-linked glycosylation sites beside a
76 signal peptide for the attachment of a glycosylphosphatidylinositol moiety (GPI-anchor) at the
77 C-terminus. These characteristics render Pfs25 very difficult to express (18,20). PfCSP, with its
78 biased codon usage and lopsided amino acid composition, only allows for a minute amount of
79 protein to be expressed in E. coli cells (34). The other antigen candidate is the PfAMA1 gene,
80 which codes for a type-1 integral membrane protein of merozoites and is also difficult to express.
81 Only a synthetic and codon optimized gene has produced a fairly large amount of PfAMA1 protein
82 in E. coli cells. Furthermore, a series of labor-intensive and technically complex refolding
83 processes of the aggregates was required to use the protein as an antigen (6). The fact that only a
84 handful of vaccine candidates are currently available (23) is most likely the result of difficulties in
85 expressing malarial antigens in high quantity with their correct conformation.

86 We previously developed a wheat germ cell-free protein synthesis system for practical use
87 in protein production. The system is especially powerful when used for the production of
88 eukaryotic proteins because of its eukaryotic nature. We established two wheat germ cell-free
89 protocols for practical use. The first can be used to produce a small amount of protein from a large
90 number of cDNAs, in parallel, for the examination of product qualities and for the genome-wide
91 biochemical annotation of gene products. In this approach, the templates for transcription are
92 constructed using the split-PCR approach (29). The solution resulting from transcription is then

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93 directly used as the mRNA source in the small-scale bilayer translation system (28). The second
94 protocol enables the production of large quantities of proteins. In this case, suitable gene products
95 are first selected using the small-scale parallel production method and subsequent functional
96 screening. Genes of interest are then cloned into the pEU plasmid (29) and the mRNA transcribed.
97 In the translational step, the protein production employs either the bilayer or discontinuous batch
98 translation method. The bilayer method has acceptable performance for the production of hundreds
99 of micrograms of protein. Since 150 mg of a control protein in a reaction volume of 50 ml was
100 produced in 5 h with the latter reaction method, the cell-free method can be scaled up (27). The
101 system has been acknowledged in the fields of structural and functional genomics of eukaryotes
102 (7,32) and has proved advantageous due to its capacity to yield good quality proteins. Taken
103 together, the system seems to be powerful when used for the production of malaria parasite
104 proteins, as no glycosylation takes place during the standard reaction. However, to date, there is no
105 Good Manufacturing Practice facility for production of recombinant proteins for clinical studies
106 using the wheat germ cell-free system in the world. In the present study, we first tested the
107 versatility of the wheat germ cell-free system using as control models the leading vaccine
108 candidate genes from P. falciparum. In addition, a series of experiments was conducted to prove
109 the value of the system for the parallel expression of malaria proteins. The results presented here
110 suggest that the wheat germ cell-free system may be useful as an additional protein production
111 method in the field of P. falciparum research.
112
113 ## MATERIALS AND METHODS

114 **Genomic cloning and construction of genes encoding fragments of Pfs25, PfCSP, and**
115 **PfAMA1.** The nucleotide sequences for the signal peptide and the GPI-anchor were excluded from
116 the expression constructs for genes encoding the PfCSP and Pfs25 proteins. The truncated versions

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117 of the PfCSP and Pfs25-3D7 genes were amplified by PCR from the genomic DNA of the P.
118 falciparum 3D7 strain, and subcloned into pEU3 (a vector carrying the C-terminus His_6-tag) (29) at
119 the EcoRV site. The gene encoding Pfs25-TBV was a generous gift from Dr. Anthony W. Stowers
120 (NIAID, NIH, Rockville, MD) (35). Pfs25-TBV, a synthetic version of the Pfs25 gene, was
121 codon-optimized for expression in the yeast, Saccharomyces cerevisiae, and the substitution of
122 Asn by Gln at three N-glycosylation sites was performed (20). DNA encoding full length PfAMA1
123 protein was amplified from the genomic DNA of P. falciparum 3D7 and cloned into
124 pEU-E01-GST (a vector with an N-terminal GST-tag followed by a TEV protease cleavage site)
125 between the XhoI/BamHI sites. These pEU plasmid vectors are the expression vectors designed
126 specifically for the wheat gem cell-free system (16). The inserted nucleotide sequences were
127 confirmed using the ABI PRISM® 310 Genetic Analyzer and the BigDye Terminator v1.1 Cycle
128 Sequencing kit (Applied Biosystems, Foster City, CA).
129 **Parallel construction of the DNA template from the parasite RNA.** We selected 124
130 genes annotated as dominantly expressed in the blood stages of P. falciparum based on the
131 microarray data integrated in the PlasmoDB database (http://www.plasmodb.org) (Supplement
132 Table S1). Extracted total RNA from P. falciparum 3D7 asexual blood-stage parasites was
133 reverse-transcribed into cDNA by SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad,
134 CA) and PCR amplification was performed using LA Taq™ DNA polymerase (Takara Bio, Otsu,
135 Japan). The 5′-primers were designed as 46-mers; 16-mer nucleotides
136 (5′-CCACCCACCACCACCA) as the S1-tag sequence followed by a 30-mer of unique sequence
137 covering each 5′ open reading frame containing the start codon. For the 3′-primers, 30-mer
138 nucleotides covering each unique sequence upstream from the termination codon were prepared.
139 The PCR products were then cloned into the pCR2.1 plasmid using a TOPO TA cloning kit
140 (Invitrogen) and their sequences at both ends confirmed. Translation templates were prepared by in

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141 vitro transcription from PCR products amplified by the split-primer PCR method described earlier
142 (29).

143 **Production and purification of the Pfs25-3D7/WG, Pfs25-TBV/WG, PfCSP/WG and**
144 **PfAMA1/WG proteins.** We employed the wheat germ cell-free protein expression system for
145 protein production using the bilayer translation reaction method described previously (28). Briefly,
146 250 μl of transcription mixture containing 25 μg of the plasmid DNA, 80 mM HEPES-KOH, pH
147 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM DTT, 2.5 mM each of NTPs, 250 U of
148 SP6 RNA polymerase (Promega, Madison, WI), and 250 U of RNasin (Promega) were incubated
149 for 6 h at 37°C. After the incubation, the transcription solution containing transcribed mRNA was
150 mixed with 250 μl of wheat germ extract (60 A_{260} units), supplemented with 2 μl of 20 mg/ml
151 creatine kinase in a single well of a 6-well plate. The 5.5-ml substrate mix (30 mM HEPES-KOH,
152 pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT,
153 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate) from the
154 ENDEXT® Wheat Germ Expression S Kit (CFS Co., Ltd., Matsuyama, Japan) was then added on
155 top of the translation mix and incubated at 26°C for 12 h. After incubation, the reaction mixture was
156 centrifuged at 21,900 × g for 20 min. Recovered supernatants were passed through Amicon Ultra
157 Centrifugal Filter Units (10 kDa molecular weight cut off)(Millipore, Billerica, MA) to replace the
158 translation buffer with phosphate-buffered saline. The samples containing the synthesized
159 Pfs25-3D7/WG, Pfs25-TBV/WG, and PfCSP/WG proteins were purified using the
160 Ni-NTA-agarose column (Qiagen,Valencia, CA). The PfAMA1/WG protein was purified by
161 passing the supernatant through the glutathione-Sepharose 4B column (GE Healthcare
162 Bio-Sciences, Piscataway, NJ), followed by TEV protease (Invitrogen) cleavage to remove the
163 GST tag. Concentrations of affinity-purified proteins were determined using the Bradford Protein
164 Assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples were separated by

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165 SDS-polyacrylamide gel electrophoresis under reducing conditions (22), and the bands were

166 visualized with Coomassie Brilliant Blue. Purified protein samples were stored in aliquots at

167 –80°C until further use. For parallel protein synthesis from 124 malaria genes, the transcription and

168 translation reactions were performed in a similar method to that described above. The 125-µl

169 substrate mixture was overlaid on top of the 25-µl translation mixture containing transcribed

170 mRNA in the presence of U-[^{14}C]Leu (11.1 kBq; 15 GBq/mmol of Leu). The reaction was

171 performed in 96-well plates. Proteins were separated by SDS-PAGE and identified by

172 autoradiography using an imaging analyzer (BAS-2500; Fujifilm, Tokyo, Japan). The solubility of

173 each product was expressed as the percentage of TCA-insoluble radioactivity [counted using a

174 liquid scintillation counter (LSC-6100; Aloka, Mitaka, Japan)] in a supernatant fraction recovered

175 from centrifugation at 21,900 × g for 20 min compared to that of the total reaction mixture. The

176 amount of target protein was estimated using the following formula where Count is the

177 radioactivity of the protein produced; Leu is the number of Leu residues in the protein, used to

178 estimate the moles of Leu incorporated, Mw is molecular weight, and Ratio is the ratio of intensity

179 of a specific protein band to the total intensity of bands on the autoradiogram.

180 Protein concentration = Count ÷ Leu × Mw × Ratio

181 **Preparation of antiserum.** Groups of female BALB/c mice (5 mice in each group) were

182 subcutaneously immunized three times on the 1st, 3rd and 5th weeks with 10 µg of affinity-purified

183 proteins emulsified in Freund's adjuvant. As the control, a group of mice was administered GST in

184 Freund's adjuvant, using the same protocol as described above. Antiserum preparation was as

185 described elsewhere (2).

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186 **Preparation of P. falciparum asexual blood-stage parasites, ookinetes, and sporozoites.**
187 A mature schizont-rich fraction was obtained from cultured P. falciparum 3D7 strain (30). Parasite
188 pellets were kept at −80°C until extract preparation.

189 To obtain ookinetes and sporozoites of P. falciparum, we used parasites derived from
190 patient blood. The use of all human materials in this study was reviewed and approved by the
191 Institutional Ethics Committee of the Thai Ministry of Public Health and the Human Subjects
192 Research Review Board of the United States Army. Peripheral blood was collected by heparinized
193 syringes under written informed consent from patients who came to the malaria clinics in the Mae
194 Sod district, Thailand. Infection with P. falciparum was confirmed by the microscopic observation
195 of Giemsa-stained thick and thin blood smears. The gametocytemic patient blood was divided into
196 two parts. One was used to grow zygotes/ookinetes in vitro for both western blotting and
197 immunocytochemical analyses, and the other half was subjected to propagation of sporozoites in
198 mosquitoes for two further analyses, as described elsewhere (33). Western blot analysis and
199 immunocytochemistry were performed as described previously (3,17).

200 **Transmission-blocking assays.** We collected 20 ml of peripheral blood from a volunteer
201 patient. Blood was divided into aliquots (300 μl/tube), briefly centrifuged and plasma was discarded.
202 Mouse immune sera against both Pfs25-3D7/WG and Pfs25-TBV/WG were serially diluted with
203 heat-inactivated normal human serum prepared from malaria-naïve donors. Next, 180 μl of each
204 diluted solution was added to the P. falciparum-infected blood cells and incubated for 15 min at
205 room temperature. The mixture was placed in a membrane feeding apparatus kept at 37°C to allow
206 Anopheles dirus A mosquitoes to feed on the blood in each apparatus for 30 min. Fully engorged
207 mosquitoes were maintained for a week in the insectary. Oocysts that developed within the midgut
208 were counted from 20 randomly selected mosquitoes. The Kruskal-Wallis test was applied to
209 examine the differences in oocyst counts per mosquito between immunized groups and the control

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210 group fed on mouse serum raised against GST. Probability values (P) less than 0.05 were
211 considered statistically significant.
212

213 **RESULTS AND DISCUSSION**

214 We were able to successfully express the Pfs25/WGs, PfCSP/WG, and PfAMA1/WG
215 proteins using the wheat germ cell-free system. Expression of the Pfs25 (Pfs25-3D7/WG) protein
216 from a gene with a native nucleotide composition was shown by subsequent SDS-PAGE analysis
217 (Fig. 1A) to be comparable in amount to that of Pfs25-TBV/WG (Fig. 1B) expressed from the
218 codon-optimized synthetic gene. On the SDS-PAGE gels, two protein bands appeared at 20 kDa,
219 the expected mobility of the Pfs25 truncated form, lacking the signal peptide and the GPI-anchor.
220 Almost all of the Pfs25-3D7/WG protein from the biased DNA was recovered in the supernatant
221 fraction (Lane A2), and was easily purified as a single dominant band along with other nonspecific
222 faint bands by affinity chromatography (Lane A5). The amount of purified Pfs25-3D7/WG was 35
223 μg per 6.0 ml of the reaction mixture, while that obtained from the codon-optimized gene was
224 comparable, at 30 μg protein per reaction. These results demonstrate that the wheat germ cell-free
225 system we employed produced an equal amount of protein with or without prior optimization of
226 their biased codon usage in the DNA. Similarly, the amount of the other two proteins, PfCSP/WG
227 (Fig. 1C), and PfAMA1/WG (Fig. 1D), produced from a gene with a native nucleotide composition
228 were 26 and 102 μg per reaction, respectively.

229 **Immunological characterization of the protein products.** To determine the creation of
230 conformation-dependent epitopes in Pfs25 and AMA-1, we examined and confirmed the reactivity
231 of anti-Pfs25 conformation-specific monoclonal antibody, 4B7 [a generous gift from Dr. Carole A.
232 Long (NIAID, NIH, Rockville, MD)], against Pfs25/WGs, and the reactivity of anti-PfAMA1 3D7
233 conformation-specific monoclonal antibody, 1E9 (a generous gift from Dr. Carole A. Long), against

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234 PfAMA1/WG by western blotting under non-reducing conditions (data not shown). To evaluate the
235 immunogenicity of each protein prepared, we then raised mouse antisera and determined their
236 reactivity to the parasite-derived native proteins. Extract from approximately 5 x 10^5
237 zygotes/ookinetes per lane was separated by SDS-PAGE and western blot analysis was performed.
238 Specific bands with the expected mobility of native Pfs25 protein were detected under non-reducing
239 conditions using antisera against Pfs25-3D7/WG and Pfs25-TBV/WG. Anti-Pfs25-3D7/WG serum
240 did not show any reactivity under reducing condition (Fig. 2A). These results suggest that the
241 Pfs25-3D7/WG protein prepared here retained a conformation similar to the native protein. The
242 identity of the faint band detected at the lower position with anti-Pfs25-TBV/WG under reducing
243 condition is unclear at present (Fig. 2A). Similar experiments were performed using
244 anti-PfCSP/WG and anti-PfAMA1/WG sera to study extracts from respective stages of the parasite.
245 The analyses clearly showed specific reactivity of each antiserum to PfCSP and PfAMA1 proteins
246 (Figs. 2B and 2C). Anti-PfCSP serum reacted to three protein bands in the sporozoite extract under
247 both reducing and non-reducing conditions (Fig. 2B). The upper and lower bands appeared to
248 correspond to precursor and mature forms, respectively, as reported earlier by Coppi et al. (4).
249 Anti-PfAMA1 serum gave two signals, with the upper and lower bands corresponding to mature
250 and processed forms, respectively (15). The signal shift of the two bands upon introduction of a
251 reducing reagent was most likely due to the high content of disulfide bonds within the protein (14).
252 These results are consistent with previously reported findings (13).
253 Immunocytochemical staining was performed against immature ookinetes obtained by in
254 vitro short-term culture using anti-Pfs25-3D7/WG. As shown in Figure 2D (DIC and IFA), the
255 antiserum specifically stained the surface of immature ookinete but not gametocyte and merozoite.
256 Antiserum against Pfs25-TBV/WG yielded similar results (data not shown). These findings were
257 consistent with our previous report in which Pfs25-TBV prepared from yeast cells was used to raise

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258 antiserum (2). These findings also verified that Pfs25 prepared using our protocols from a gene with
259 an A/T-rich native nucleotide composition can yield a protein of sufficient quality to raise a specific
260 antibody. Experiments using anti-PfCSP/WG and anti-PfAMA1/WG on the target stages of the
261 parasite showed typical staining patterns. The entire surface of the slender-shaped sporozoite was
262 stained by anti-PfCSP/WG serum (Fig. 2E), and the anti-PfAMA1/WG serum clearly visualized
263 punctate localization of PfAMA1 at the apical end of merozoites (Fig. 2F).

264 **Efficacy evaluation of the proteins as vaccine antigens.** In view of a practical application
265 of the system for discovery of malaria vaccine candidates, we evaluated the quality of antigens
266 produced by performing a parasite growth inhibition assay using the antibodies raised against those
267 antigens. We focused on Pfs25-3D7/WG and Pfs25-TBV/WG. Pfs25-TBV is currently the sole
268 transmission-blocking vaccine candidate under clinical trial (23). A transmission-blocking assay
269 was performed using both anti-Pf25-3D7/WG and anti-Pfs25-TBV/WG. A mixture containing P.
270 falciparum-gametocyte infected erythrocytes and one of the antisera was fed to mosquitoes. The
271 number of developed oocysts in the mosquitoes was then later counted. Both antisera at two-fold
272 dilution completely inhibited oocyst development, as we have seen no mosquito harboring oocysts
273 (Fig. 3). The number of oocysts was inversely proportional to the concentration of antiserum added,
274 findings consistent with previous experiments, in which Pfs25-TBV prepared from yeast was used
275 to raise antiserum (2). It is important at this moment to stress the difference between this study and
276 other studies: our proteins were produced from a codon-non-optimized gene in a cell-free system,
277 while in other studies a codon-optimized engineered Pfs25-TBV gene was transformed into yeast
278 cells (20). The results presented here strongly indicate the value of the wheat germ cell-free system
279 for the production of malaria proteins that require complicated procedures in other systems.

280 **Parallel syntheses of P. falciparum proteins.** Although cell-based expression systems
281 have been widely used in this field, they are limited mainly in their ability for efficient production

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282 of P. falciparum protein, primarily because of the complexity of the genome. In order to evaluate
283 the capability of our cell-free system for parallel expression from the parasite genes, we selected
284 124 genes (Supplement Table S1) encoding asexual blood-stage parasite proteins, based on the
285 PlasmoDB. Autoradiography demonstrated that 93 of the 124 genes yielded protein products. The
286 average yield of expressed protein estimated for each full size product was 1.9 μg per 150 μl of
287 reaction mixture, an amount sufficient for preliminary antigen discovery studies using
288 hyper-immune serum. Average protein solubility was 65% (Supplement Table S1). There was
289 significant inverse correlation between yield and molecular size of the protein; the greater the size,
290 the lower the protein yield. There was also weak but significant inverse correlation between the
291 protein yield and relative frequency of low complexity regions. In addition, solubility was
292 inversely correlated with the pI-value (Table 1). These observations have already been documented
293 in earlier studies (24,31). Surprisingly, we did not see any correlation between either yield or
294 protein solubility and A/T content, pI-value, or the existence of a transmembrane domain (data not
295 shown). We then analyzed the statistical difference in molecular weights, pI-values, A/T contents,
296 and relative frequency of low complexity regions between the expressed and not-expressed groups
297 of molecules, using the Mann Whitney U-test. The molecular weights in the non-expressed group
298 were significantly higher than those of the expressed group ($P < 0.0001$). In contrast, pI-values,
299 A/T contents, and the relative frequency of low complexity regions did not differ significantly
300 (Supplement Table S1). We currently have no explanation for why 25% of the tested genes failed
301 to produce proteins in our system. One possible explanation is the sequence errors most likely
302 present in the PCR products used as templates for transcription and subsequent translation. Such
303 templates would cause mistranslation of the protein by frame shift.

304 In summary, the ability of the wheat germ cell-free protein synthesis system to produce P.
305 falciparum proteins was examined. We found that: 1) without the need for codon optimization, the

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306 cell-free system is able to produce a sufficient amount of good quality proteins of the leading
307 malaria vaccine candidates, Pfs25, PfCSP, and PfAMA1; 2) biochemical, immunocytochemical,
308 and biological analyses demonstrated that the prepared proteins could be directly used for
309 immunization after a simple affinity purification step; and 3) the system proved suitable for use as
310 a parallel way to produce parasite proteins. We believe that the wheat germ cell-free protein
311 synthesis system may be a key tool for decoding genetic information above and beyond malaria
312 vaccine research.
313

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