75. VanBuskirk KM, O'Neill MT, De La Vega P, et al. Preerythrocytic, live-attenuated plasmodium falciparum vaccine candidates by design. Proc Natl Acad Sci USA 2009: 106;13004-9

76. Cao J, Kaneko O, Thongkukiatkul A, et al. Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in *plasmodium falciparum* merozoites. Parasitol Int 2009;58:29-35

77. Otsuki H, Kaneko O, Thongkukiatkul A, et al. Single amino acid substitution in *plasmodium yoelii* erythrocyte ligand determines its localization and controls parasite virulence. Proc Natl Acad Sci USA 2009;106:7167-72

78. Dutta S, Lalitha PV, Ware LA, et al. Purification, characterization, and immunogenicity of the refolded ectodomain of the *plasmodium falciparum* apical membrane antigen 1 expressed in escherichia coli. Infect Immun 2002;70:3101-10

79. Netzer WJ, Hartl FU. Recombination of protein domains facilitated by co-translational folding in eukaryotes. Nature 1997;388:343-9

80. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002;295:1852-8

Affiliation

Satoru Takeo[1], Thangavelu U Arumugam[1], Motomi Torii[2] & Takafumi Tsuboi[†1] MD
[†]Author for correspondence
[1]Professor,
Ehime University,
Cell-free Science and
Technology Research Center, 3 Bunkyo-cho,
Matsuyama, 790-8577, Japan
Tel: +81 89 927 8277; Fax: +81 89 927 9941;
E-mail: tsuboi@ccr.ehime-u.ac.jp
[2]Ehime University Graduate School of Medicine,
Department of Molecular Parasitology,
454 Shitsukawa, Toon, 791-0295, Japan.

INFECTION AND IMMUNITY, Dec. 2009, p. 5496–5500 Vol. 77, No. 12
0019-9567/09/$12.00 doi:10.1128/IAI.00640-09

Malaria Ookinete Surface Protein-Based Vaccination via the Intranasal Route Completely Blocks Parasite Transmission in both Passive and Active Vaccination Regimens in a Rodent Model of Malaria Infection▽

Takeshi Arakawa,[1,2] Mayumi Tachibana,[3] Takeshi Miyata,[1] Tetsuya Harakuni,[1] Hideyasu Kohama,[1] Yasunobu Matsumoto,[4] Naotoshi Tsuji,[5] Hajime Hisaeda,[6]† Anthony Stowers,[6]‡ Motomi Torii,[3] and Takafumi Tsuboi[7]*

Molecular Microbiology Group, COMB, Tropical Biosphere Research Center, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan[1]; Division of Host Defense and Vaccinology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan[2]; Department of Molecular Parasitology, Ehime University School of Medicine, Shigenobu-cho, Ehime 791-0295, Japan[3]; Laboratory of Global Animal Resource Science, Department of Global Agricultural Sciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan[4]; National Institute of Animal Health, National Agricultural Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan[5]; Malaria Vaccine Development Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland[6]; and Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan[7]

Received 5 June 2009/Returned for modification 6 July 2009/Accepted 6 September 2009

Malaria vaccines based on ookinete surface proteins (OSPs) of the malaria parasites block oocyst development in feeding mosquitoes and hence disrupt the parasite life cycle and prevent the disease from being transmitted to other individuals. To investigate whether a noninvasive mucosal vaccination regimen effectively blocks parasite transmission in vivo, *Plasmodium yoelii* Pys25, a homolog of the Pfs25 and Pvs25 OSPs of *Plasmodium falciparum* and *Plasmodium vivax*, respectively, was intranasally (i.n.) administered using a complement-deficient DBA/2 mouse malaria infection model, in which a highly elevated level of oocysts develops in feeding mosquitoes. Vaccinated mice developed a robust antibody response when the vaccine antigen was given together with cholera toxin adjuvant. The induced immune serum was passively transferred to DBA/2 mice 3 days after infection with *P. yoelii* 17XL, and *Anopheles stephensi* mosquitoes were allowed to feed on the infected mice before or after serum transfusion. This passive immunization completely blocked oocyst development; however, immune serum induced by the antigen or adjuvant alone did not have such a profound antiparasite effect. Further, when i.n. vaccinated mice were infected with the parasite and then mosquitoes were allowed to directly feed on the infected mice, complete blockage of transmission was again observed. To our knowledge, this is the first time that mucosal vaccination has been demonstrated to be efficacious for directly preventing parasite transmission from vaccinated animals to mosquitoes, and the results may provide important insight into rational design of nonparenteral vaccines for use against human malaria.

Malaria is one of the most important infectious diseases, and the levels of mortality and morbidity are high, especially among children in developing countries in Africa, Asia, and South America. Implementation of malaria control measures, such as antimalaria drug chemotherapy and insecticide-treated bed nets, has made a significant contribution to reducing the incidence of malaria in many parts of the world. However, these control measures may not be sufficient, and therefore new tools, including vaccines, should be included in a new malaria control campaign for local elimination and final eradiation of malaria from the globe (7). A promising strategy to counteract global malaria endemicity is to develop highly efficacious vaccines, and several promising candidates have been intensively investigated (7, 20); vaccines targeting asexual stages (i.e., sporozoite, hepatic, and erythrocytic stages) are designed to prevent infection and reduce disease severity, while vaccines that target the sexual stage, in which the parasite undergoes sporogonic development in anopheline mosquitoes, prevent vector-mediated transmission of the parasite from person to person (4, 8, 14, 17, 25). Although transmission-blocking vaccines do not directly prevent infection, they reduce parasite infectivity for the vector and consequently lower the mosquito infection rate and the frequency of transmission to humans. In addition, this strategy is believed to be particularly useful for controlling escape of mutants from vaccines designed based on antigens expressed at an asexual stage; therefore, transmission-blocking vaccines are increasingly being considered indispensable components of malaria vaccine strategies and are key components of malaria elimination (10, 11).

* Corresponding author. Mailing address: Cell-Free Science and Technology Research Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan. Phone: 81-89-927-8277. Fax: 81-89-927-9941. E-mail: tsuboi@ccr.ehime-u.ac.jp.

† Present address: Department of Immunology and Parasitology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Fukuoka 812-8582, Japan.

‡ Present address: CSL Limited A.C.N. 051 588 348, 45 Poplar Road, Parkville, Victoria 3052, Australia.

▽ Published ahead of print on 14 September 2009.

Studies on rodent and human malaria concluded that an effector mechanism that is pivotal for blocking transmission is induction of antigen-specific serum antibodies in a vaccinated host, from which female mosquitoes, when they bite to obtain blood meal, coingest gametocyte pairs together with the induced antibodies (2, 3, 5, 9, 18, 24). The ingested antibodies seem to be stable in the mosquito midgut, at least in the time frame within which the transmitted gametocytes develop into ookinetes.

Parasite antigens expressed later at the postfertilization stage in the mosquito midgut, such as ookinete surface proteins (OSPs), including Pfs25 and Pvs25 from *Plasmodium falciparum* and *Plasmodium vivax*, respectively, are particularly important vaccine targets because they are likely to be concealed immunologically, if not concealed completely, from the mammalian host's immunosurveillance system, which suggests that there is a reduced driving force to produce the antigenic variations often observed for antigens expressed at prefertilization stages (6, 12, 23, 28). In addition, several recent studies indicated that in the malaria life cycle the ookinete-to-oocyst transition stage is one of the most vulnerable stages of parasite development, making the postfertilization stage of sporogonic development an ideal target for antitransmission vaccines.

The vast majority of pathogens invade through mucosal tissues and therefore can be controlled effectively by mucosal vaccines rather than parenteral vaccines. Notwithstanding the great merit of mucosal vaccines, most vaccines in use today are delivered parenterally (subcutaneously [s.c.] or intramuscularly). In spite of many arguments against the concept that vaccines against arthropod vector-borne human pathogens, such as malaria parasites, could be designed based on mucosal delivery, recent studies performed by us and other workers demonstrated that mucosal vaccines could be efficacious for prevention of arthropod-transmitted infections, because mucosal administration of foreign antigens mixed with a potent mucosal adjuvant, such as cholera toxin (CT), can induce strong systemic immunity (2, 3, 13). Here we extended our previous studies to test our hypothesis that the malaria OSPs are sufficiently immunogenic when they are administered by the intranasal (i.n.) route in the presence of a mucosal adjuvant, which should in theory effectively block parasite transmission to feeding mosquitoes when both passive and active vaccination regimens are used.

MATERIALS AND METHODS

Mice, vaccination, and antibody enzyme-linked immunosorbent assay (ELISA). Seven-week-old female DBA/2NCrj (DBA/2) mice were purchased from Japan SLC (Tokyo, Japan). Complement C5-deficient DBA/2 mice were used for live mosquito-feeding experiments, because the highly elevated levels of oocysts that developed in the mosquito midgut were useful for evaluation of transmission-blocking vaccine efficacy (26).

Mice were i.n. vaccinated once a week for 4 weeks with 25 μg of yeast-derived recombinant *Plasmodium yoelii* Pys25 synthesized and purified like Pvs25 as described previously (14) in the absence or presence of 1 μg of CT (Sigma-Aldrich). As a control, a group of mice were vaccinated with 1 μg of CT alone. For passive vaccination experiments, DBA/2 mice were intravenously vaccinated with 0.5 ml of pooled immune sera derived from mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone.

For ELISA of vaccine-induced immune sera, a flat-bottom 96-well microtiter plate (Immulon 4; Dynex Technology Inc., Chantilly, VA) was coated with recombinant Pys25 (0.5 μg/well in bicarbonate buffer, pH 9.6) and blocked with 1% skim milk in Tris-buffered saline containing 0.05% Tween 20. Immune sera serially diluted with the blocking buffer were applied to wells in duplicate (100 μl/well) and incubated for 2 h at 37°C, which was followed by addition of alkaline phosphatase-conjugated anti-mouse antibody for immunoglobulin (Ig) isotype and IgG subclass analysis. The alkaline phosphatase substrate (*p*-nitrophenyl phosphate [Sigma-Aldrich]) was added, and the absorbance at 490 nm was determined with a microplate reader (Bio-Rad Laboratories). The antibody concentration was determined based on known amounts of mouse Igs used as a standard. The statistical significance of differences in antibody concentration or absorbance was determined by Student's *t* test.

Parasite infection, blood feeding experiment, and assay of transmission blocking. For analysis of the parasite-killing effect of i.n. vaccination-induced immune sera, mice were intraperitoneally inoculated with 10^6 peripheral red blood cells that had been infected with *P. yoelii* strain 17XL, and the infected mice were maintained for 3 days until the level of parasitemia reached 9 to 10%, which was determined by microscopic examination of Giemsa-stained thin blood smear preparations. Then approximately 100 *Anopheles stephensi* mosquitoes that had been starved overnight were allowed to obtain a blood meal from the infected mice either before or 1 h after intravenous injection of immune sera that had been prepared from mice 1 week after the last i.n. vaccination with Pys25 plus CT, with Pys25 alone, or with CT alone. Fully engorged mosquitoes were maintained at 24°C for 1 week by giving them water containing 1.5% fructose and 1.5% sucrose. For each experimental group, mosquitoes were dissected, and their midguts were examined with a light microscope to count the number of oocysts.

For analysis of the direct parasite transmission-blocking efficacy of i.n. vaccination, mice vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone were infected as described above with the parasite 1 week after the last vaccination, and then mosquitoes were allowed to feed directly on the infected animals; this was followed by enumeration of the oocysts that developed.

The statistical significance of differences in the numbers of oocysts was determined by the Kruskal-Wallis test or the Wilcoxon-Mann-Whitney U test by using the software JMP (SAS Institute Inc.).

RESULTS

A significant level of specific serum IgG and IgM antibodies (mainly IgG) was induced in DBA/2 mice by i.n. vaccination with Pys25 plus CT (14,147 ± 4,241 μg/ml) but not by i.n. vaccination with Pys25 alone or CT alone (Fig. 1a, upper panel). Oral inoculation of 50 μg of Pys25, however, did not induce an antibody response even in the presence of 10 μg CT (data not shown). IgG1 was found to be the predominant serum IgG subclass, and almost no IgG2a was detected in mice vaccinated with Pys25 plus CT, an indication of the Th2 type of immune response induction (Fig. 1a, lower panel). Low but detectable levels of Pys25-specific serum IgA and IgE were seen in the group vaccinated with Pys25 plus CT but not in the group vaccinated with Pys25 alone or CT alone (Fig. 1b). Similar humoral immune responses were observed when outbred ddy mice were used for the immunization experiments (data not shown).

To evaluate the parasite transmission-blocking effect of the induced immune sera in vivo, *A. stephensi* mosquitoes were allowed to obtain a blood meal from DBA/2 mice that had been infected with *P. yoelii* 17XL before or after passive transfer of the immune sera of mice vaccinated i.n. with Pys25 plus CT, with Pys25 alone, or with CT alone as described in Materials and Methods. For all three immunization groups, large numbers of oocysts were observed in the mosquito midgut when the mosquitoes were allowed to feed before the immune sera were transferred (median for CT alone, 346 oocysts; median for Pys25 alone, 302 oocysts; median for Pys25 plus CT, 311 oocysts) (Fig. 2a). In contrast, when the mosquitoes received the blood meal after the immune sera were transferred, oocyst formation was completely blocked in the group vaccinated with Pys25 plus CT, but not in the group vaccinated with

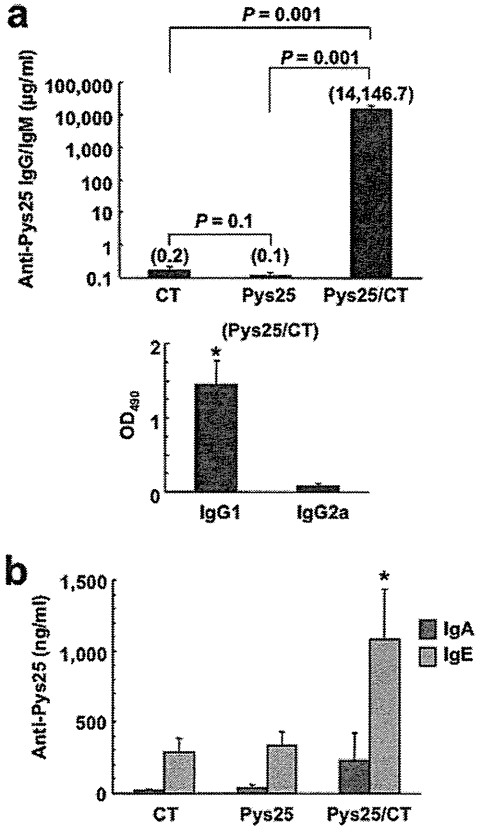

FIG. 1. Antibody responses in mice i.n. vaccinated with recombinant Pys25. DBA/2 mice (three or four mice/group) were vaccinated i.n. with a mixture of Pys25 (25 μg) and CT (1 μg), with Pys25 alone (25 μg), or with CT alone (1 μg) once a week for 4 weeks, and 1 week after the final vaccination serum antibody titers were analyzed by ELISA. (a) (Upper panel) Pys25-specific serum IgG and IgM (predominantly IgG) levels expressed as mean antibody concentrations in the serum. The error bars indicate standard deviations. (Lower panel) Immune sera (1/128,000) from mice vaccinated with Pys25 plus CT were also analyzed for the presence of IgG1 and IgG2a, and the results were expressed as mean optical densities at 490 nm (OD_{490}). The error bars indicate standard deviations. *, $P = 0.002$ for comparison of IgG1 and IgG2a. (b) Pys25-specific serum IgA and IgE levels expressed as mean antibody concentrations in serum (ng/ml). The error bars indicate standard deviations. *, $P < 0.01$ for a comparison of Pys25 plus CT and CT or Pys25. No significant differences between groups were observed for serum IgA. Statistical significance was determined by Student's *t* test.

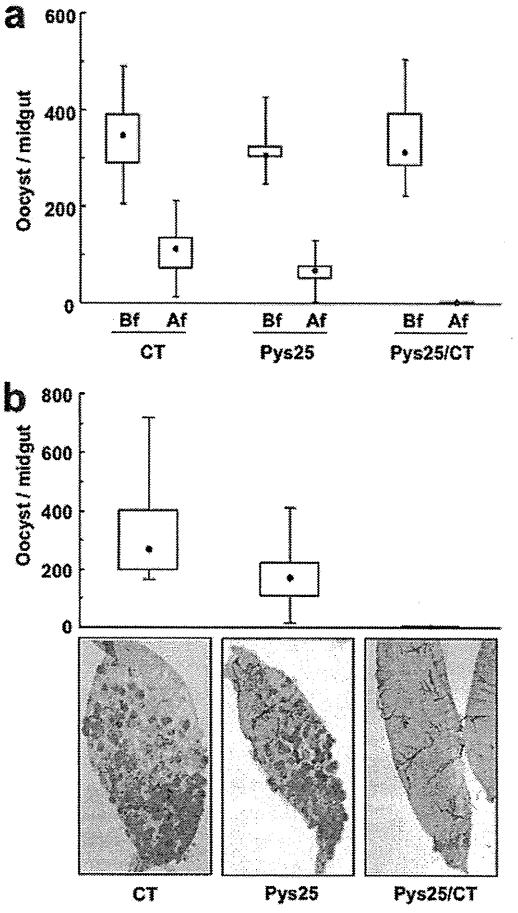

FIG. 2. Transmission-blocking vaccine efficacy. (a) Transmission-blocking effects of passively transferred immune sera on *P. yoelii* oocyst development in the *A. stephensi* mosquito midgut. Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before (Bf) or after (Af) intravenous injection of immune sera derived from mice i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone. Data are expressed as the median numbers of oocysts per mosquito (dots in boxes), quartiles (boxes), and ranges (lines above and below boxes). Statistically significant differences were found in all three immunization groups compared for the treatment after intravenous injection of immune sera ($P < 0.0001$) but not for the treatment before intravenous injection of immune sera ($P = 0.726$) in an analysis performed using the Kruskal-Wallis test. There are also statistically significant differences between the treatment before intravenous injection of immune sera and the treatment after intravenous injection of immune sera for all three immunization groups ($P < 0.0001$, Wilcoxon-Mann-Whitney U test). (b) Transmission-blocking effects of active immunization on oocyst development. Mice were i.n. vaccinated with Pys25 plus CT, with Pys25 alone, or with CT alone and then were infected with the parasite. Mosquitoes were allowed to directly feed on infected mice, the numbers of oocysts were determined, and the data were expressed as described above for panel a. Statistically significant differences were found in all three groups compared by using the Kruskal-Wallis test ($P < 0.0001$). Light microscopic images of representative mosquito midguts from the active vaccination experiments are shown at the bottom.

CT alone (median, 109 oocysts) or with Pys25 alone (median, 65 oocysts). Although we do not know why the CT or Pys25 immune serum had a significant parasite-killing effect (for CT, 346 oocysts versus 109 oocysts; for Pys25, 302 oocysts versus 65 oocysts), no mosquitoes completely lacked oocysts when they were given CT or Pys25 immune serum (Table 1). The results demonstrated that i.n. vaccination with Pys25 plus CT induced antibodies which confer complete transmission-blocking immunity when a passive vaccination regimen is used.

Next, to evaluate the direct mucosal vaccine efficacy of Pys25, mosquitoes were allowed to obtain a blood meal directly from parasite-infected mice that had been vaccinated as described in Materials and Methods. The results demonstrated that vaccination with Pys25 plus CT completely blocked oocyst development, as we observed in the passive vaccination experiment, while significant numbers of oocysts were observed in mosquitoes that fed on CT-vaccinated mice (median, 269 oo-

TABLE 1. Prevalence of oocyst infection in *A. stephensi* mosquitoes

Type of immunization	Vaccine	No. of oocyst-positive mosquitoes/ total no. examined (% infection)[a]	No. of oocyst-positive mosquitoes/total no. examined (% infection)[a]	
			Before immune serum transfer[b]	After immune serum transfer[b]
Passive	CT		31/31 (100)	40/40 (100)
	Pys25		71/71 (100)	39/39 (100)
	Pys25 + CT		51/51 (100)	0/56 (0)
Active	CT	150/150 (100)		
	Pys25	90/90 (100)		
	Pys25 + CT	0/84 (0)		

[a] The percentages are the percentages of oocyst-positive mosquitoes based on the total numbers of mosquitoes examined.

[b] Approximately 100 mosquitoes were allowed to feed on parasite-infected mice (9 to 10% parasitemia at the time of feeding) before or after intravenous transfer of immune sera obtained from mice i.n. vaccinated with Psy25 plus CT, with Psy25 alone, or with CT alone.

cysts) or Pys25-vaccinated mice (median, 170.5 oocysts) (Fig. 2b). Vaccination with Pys25 alone had a weak but significant transmission-blocking effect compared with the effect observed for CT-vaccinated mice, suggesting that i.n. vaccination with the recombinant antigen alone might have some efficacy, even though the antibody levels for these two groups were not significantly different (Fig. 1). The oocyst prevalence was 100% for all vaccination regimens that we tested except both the passive and active Pys25-plus-CT regimens, for which the oocyst prevalence was 0% (Table 1). On the basis of our results, we concluded that i.n. vaccination with the malaria OSP was very efficacious when this OSP was combined with a mucosal adjuvant to block parasite transmission to mosquitoes.

DISCUSSION

Although most vaccines in use today are administered s.c. or intramuscularly, the advantages of mucosal vaccines are indisputable; they result in local immunity as well as systemic immunity, which, in general, is hard for parenteral vaccines to induce, and they provide a first line of defense against many infections that occur at or emanate from mucosal surfaces. They could prevent transmission of blood-borne pathogens by reuse of syringes; they may be safer and more cost-effective and thus have advantages for developing countries; and they are painless and therefore likely to be readily tolerated by small children and individuals with needle phobia (16). Although not all of the advantages attributed to mucosal vaccines mentioned above are directly relevant to the design of vaccines against malaria, and although there are some intrinsic technical difficulties that cannot be circumvented by development of effective mucosal vaccines (7), evaluation of the concept of designing mucosal vaccines for nonmucosal pathogens seems to be worthwhile.

To investigate malaria parasite OSP-based mucosal vaccines, we previously demonstrated that for two types of human malaria (*P. falciparum* malaria and *P. vivax* malaria) experimentally induced mouse immune sera specific for Pfs25 and Pvs25 (homologues of rodent Pys25) were very effective in blocking parasite transmission from patients' parasitized blood to mosquitoes in a membrane feeding assay (2, 3). In the present study we demonstrated that in a rodent malaria infection model, OSPs were immunogenic when they were administered i.n. (with levels of antigen-specific Igs reaching 15 mg/ml), and the induced immune serum was very effective in blocking parasite transmission. Most importantly, however, we demonstrated that vaccination directly prevented the transmission of a parasite from vaccinated animals to feeding mosquitoes. To our knowledge, this is the first demonstration that mucosal vaccination with malaria OSPs can directly prevent malaria transmission to mosquitoes in vivo. In the DBA/2 strain of mice lacking a component of the complement system, the number of oocysts formed is significantly increased in feeding mosquitoes (26); however, anopheline mosquitoes collected in field are usually not as heavily infected, and a single oocyst is commonly detected. Therefore, a more moderate antibody level may confer effective transmission-blocking immunity in humans (21).

Malaria vaccines targeting hepatic and erythrocytic stages suffer from antigenic variations mainly due to selection pressure from the host immune system. However, antigens expressed at a parasite sexual stage, such as OSPs, are immunologically concealed from the host immune system, and hence the chance that antigenic variations occur may be low. Indeed, OSPs of *P. falciparum* and *P. vivax* were shown to have minimal antigenic variations even in field isolates collected from remote regions of the world (19, 27). This is an important characteristic of ideal vaccines. On the one hand, sexual-stage antigens have disadvantages such as (i) the absence of an infection-induced booster effect and the resulting long-term immunity and (ii) the absence of direct protection of vaccinees from infection. Therefore, it is believed that a vaccine candidate should have multiple components and that at least one component should be a sexual-stage antigen (7). In such a vaccine formulation, preerythrocytic and/or erythrocytic antigens may function cooperatively with sexual-stage antigens for prevention of or reduction of infection and parasite transmission.

Mucosal administration, such as i.n. or oral administration, unlike parenteral immunization, of nonreplicating inert antigens with CT tends to induce Th2-type immunity, which is characterized by predominant induction of serum IgG1, induction of local secretory IgA, and in some cases induction of serum IgE in mouse models. Unlike what happens in other infectious diseases, which require induction of cell-mediated immunity (22), serum antibody, regardless of the Ig isotype, seems to be the predominant, if not only, protective arm of immunity that blocks malaria transmission. We do not know the mechanism of action of Pys25-specific antibodies in blocking parasite development in the midgut of a feeding mosquito, but binding of antibodies to the zygote surface and subsequent prevention of parasite development into the ookinete may be the most important blocking mechanism (14, 25). This antibody binding may occur within the midgut, and this may be independent of Ig isotypes. Thus, although IgE antibody is not the major antibody isotype present in a vaccinated host, it may contribute to blocking transmission. However, the induction of serum IgE may potentially lead to an allergic response in vaccinated individuals, and the data shown in Fig. 1b are relevant to this issue. Another important issue that needs to be

considered is the duration of protective antibodies. Recent findings relevant to the present work indicated that when *P. vivax* transmission-blocking vaccine candidate Pvs25 was injected s.c. with incomplete Freund's adjuvant into BALB/c mice, it induced a strong antigen-specific serum IgG response that was maintained for more than 6 months (our unpublished data). i.n. vaccination with Pvs25 plus CT induced a level of serum IgG comparable to that induced by s.c. vaccination formulated with incomplete Freund's adjuvant, but the level gradually decreased over 6 months. However, we found that i.n. vaccination with Pvs25 plus CT was generally more potent based on the magnitude and duration of the specific serum IgG response than s.c. vaccination with Pvs25 formulated with aluminum hydroxide (unpublished data).

In this study we used CT as a mucosal adjuvant; however, the use of CT for humans is hampered by the toxicity of this compound. Also, as mentioned above, issues related to the potential allergic response and the duration of antibodies need special consideration. Fortunately, however, nontoxic and thus safer adjuvants, but adjuvants that are as effective as CT, are being developed, making a mucosal malaria vaccine a feasible goal (1, 15). For example, we recently found that when a nontoxic subunit of CT, CTB, was fused to malaria OSP, it was efficacious by both the mucosal and s.c. routes for blocking parasite transmission (unpublished data). Thus, if the mucosal transmission-blocking vaccine efficacy data obtained with this rodent infection model can be reproduced in human clinical trials with guaranteed safety, OSP antigens formulated as noninvasive vaccines may become a powerful tool for use against human malaria.

ACKNOWLEDGMENTS

This work was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. It was also supported in part by Grant-in-Aid for Scientific Research 18390129 from MEXT, Japan.

REFERENCES

1. **Agren, L. C., L. Ekman, B. Löwenadler, and N. Y. Lycke.** 1997. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J. Immunol. **158:**3936–3946.
2. **Arakawa, T., A. Komesu, H. Otsuki, J. Sattabongkot, R. Udomsangpetch, Y. Matsumoto, N. Tsuji, Y. Wu, M. Torii, and T. Tsuboi.** 2005. Nasal immunization with a malaria transmission-blocking vaccine candidate, Pfs25, induces complete protective immunity in mice against field isolates of *Plasmodium falciparum*. Infect. Immun. **73:**7375–7380.
3. **Arakawa, T., T. Tsuboi, A. Kishimoto, J. Sattabongkot, N. Suwanabun, T. Rungruang, Y. Matsumoto, N. Tsuji, H. Hisaeda, A. Stowers, I. Shimabukuro, Y. Sato, and M. Torii.** 2003. Serum antibodies induced by intranasal immunization of mice with *Plasmodium vivax* Pvs25 co-administered with cholera toxin completely block parasite transmission to mosquitoes. Vaccine **21:**3143–3148.
4. **Carter, R.** 2001. Transmission blocking malaria vaccines. Vaccine **19:**2309–2314.
5. **del Carmen Rodriguez, M., P. Gerold, J. Dessens, K. Kurtenbach, R. T. Schwartz, R. E. Sinden, and G. Margos.** 2000. Characterisation and expression of pbs25, a sexual and sporogonic stage specific protein of *Plasmodium berghei*. Mol. Biochem. Parasitol. **110:**147–159.
6. **Escalante, A. A., A. A. Lal, and F. J. Ayala.** 1998. Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum*. Genetics **149:**189–202.
7. **Genton, B.** 2008. Malaria vaccines: a toy for travelers or a tool for eradication? Expert Rev. Vaccines **7:**597–611.
8. **Gozar, M. M., O. Muratova, D. B. Keister, C. R. Kensil, V. L. Price, and D. C. Kaslow.** 2001. *Plasmodium falciparum*: immunogenicity of alum-adsorbed clinical-grade TBV25-28, a yeast-secreted malaria transmission-blocking vaccine candidate. Exp. Parasitol. **97:**61–69.
9. **Gozar, M. M., V. L. Price, and D. C. Kaslow.** 1998. *Saccharomyces cerevisiae*-secreted fusion proteins Pfs25 and Pfs28 elicit potent *Plasmodium falciparum* transmission-blocking antibodies in mice. Infect. Immun. **66:**59–64.
10. **Greenwood, B.** 2008. Can. malaria be eliminated? Trans. R. Soc. Trop. Med. Hyg. **103S:**S2–S5.
11. **Greenwood, B. M., D. A. Fidock, D. E. Kyle, S. H. Kappe, P. L. Alonso, F. H. Collins, and P. E. Duffy.** 2008. Malaria: progress, perils, and prospects for eradication. J. Clin. Investig. **118:**1266–1276.
12. **Hafalla, J. C., M. L. Santiago, M. C. Pasay, B. L. Ramirez, M. M. Gozar, A. Saul, and D. C. Kaslow.** 1997. Minimal variation in the Pfs28 ookinete antigen from Philippine field isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **87:**97–99.
13. **Harakuni, T., H. Kohama, M. Tadano, G. Uechi, N. Tsuji, Y. Matsumoto, T. Miyata, T. Tsuboi, H. Oku, and T. Arakawa.** 2009. Mucosal vaccination approach against mosquito-borne Japanese encephalitis virus. Jpn. J. Infect. Dis. **62:**37–45.
14. **Hisaeda, H., A. W. Stowers, T. Tsuboi, W. E. Collins, J. S. Sattabongkot, N. Suwanabun, M. Torii, and D. C. Kaslow.** 2000. Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. Infect. Immun. **68:**6618–6623.
15. **Holmgren, J., J. Adamsson, F. Anjuère, J. Clemens, C. Czerkinsky, K. Eriksson, C. F. Flach, A. George-Chandy, A. M. Harandi, M. Lebens, T. Lehner, M. Lindblad, E. Nygren, S. Raghavan, J. Sanchez, M. Stanford, J. B. Sun, A. M. Svennerholm, and S. Tengvall.** 2005. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. Immunol. Lett. **97:**181–188.
16. **Holmgren, J., and C. Czerkinsky.** 2005. Mucosal immunity and vaccines. Nat. Med. **11:**S45–53.
17. **Kaslow, D. C.** 1997. Transmission-blocking vaccines: uses and current status of development. Int. J. Parasitol. **27:**183–189.
18. **Kaslow, D. C., C. Bathurst, T. Lensen, T. Ponnudurai, P. J. Barr, and D. B. Keister.** 1994. *Saccharomyces cerevisiae* recombinant Pfs25 adsorbed to alum elicits antibodies that block transmission of *Plasmodium falciparum*. Infect. Immun. **62:**5576–5580.
19. **Kaslow, D. C., I. A. Quakyi, and D. B. Keister.** 1989. Minimal variation in a vaccine candidate from the sexual stage of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **32:**101–103.
20. **Richie, T. L., and A. Saul.** 2002. Progress and challenges for malaria vaccines. Nature **415:**694–701.
21. **Saul, A.** 2008. Efficacy model for mosquito stage transmission blocking vaccines for malaria. Parasitology **135:**1497–1506.
22. **Seder, R. A., and A. V. Hill.** 2000. Vaccines against intracellular infections requiring cellular immunity. Nature **406:**793–798.
23. **Shi, Y. P., M. P. Alpers, M. M. Povoa, and A. A. Lal.** 1992. Single amino acid variation in the ookinete vaccine antigen from field isolates of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **50:**179–180.
24. **Stowers, A. W., D. B. Keister, O. Muratova, and D. C. Kaslow.** 2000. A region of *Plasmodium falciparum* antigen Pfs25 that is the target of highly potent transmission-blocking antibodies. Infect. Immun. **68:**5530–5538.
25. **Tsuboi, T., Y. M. Cao, Y. Hitsumoto, T. Yanagi, H. Kanbara, and M. Torii.** 1997. Two antigens on zygotes and ookinetes of *Plasmodium yoelii* and *Plasmodium berghei* that are distinct targets of transmission-blocking immunity. Infect. Immun. **65:**2260–2264.
26. **Tsuboi, T., Y. M. Cao, M. Torii, Y. Hitsumoto, and H. Kanbara.** 1995. Murine complement reduces infectivity of *Plasmodium yoelii* to mosquitoes. Infect. Immun. **63:**3702–3704.
27. **Tsuboi, T., O. Kaneko, Y. M. Cao, M. Tachibana, Y. Yoshihiro, T. Nagao, H. Kanbara, and M. Torii.** 2004. A rapid genotyping method for the vivax malaria transmission-blocking vaccine candidates, Pvs25 and Pvs28. Parasitol. Int. **53:**211–216.
28. **Tsuboi, T., D. C. Kaslow, M. M. Gozar, M. Tachibana, Y. M. Cao, and M. Torii.** 1998. Sequence polymorphism in two novel *Plasmodium vivax* ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates. Mol. Med. **4:**772–782.

Editor: W. A. Petri, Jr.

Practical cell-free protein synthesis system using purified wheat embryos

Kazuyuki Takai, Tatsuya Sawasaki & Yaeta Endo

Cell-Free Science and Technology Research Center and Venture Business Laboratory, Ehime University, Matsuyama, Japan. Correspondence should be addressed to Y.E. (yendo@eng.ehime-u.ac.jp).

Published online 21 January 2010; doi:10.1038/nprot.2009.207

Biochemical characterization of each gene product encoded in the genome is essential to understand how cells are regulated. The bottleneck has been and still is in how the gene products can be obtained. The wheat cell-free protein synthesis system we have developed is a powerful method for preparation of many different proteins at a time and also for preparation of large amounts of specific proteins for biochemical and structural analyses. Here, we show a method for preparation of the wheat embryo extract useful for the cell-free reactions, by which 5 ml of a high-activity extract is obtained in 4–5 d. We also describe the methods for small- and large-scale protein synthesis by hands-down operations with the use of mRNAs prepared by transcription of PCR products and pEU plasmids harboring the target cDNAs, which need 2–4 d excepting the time required for plasmid preparation.

INTRODUCTION

With much information on the genome and cDNA sequences of many different organisms, we can now obtain and deduce a lot of information about the gene products and their interactions with the aid of bioinformatics, genetics and cell biology. However, such pieces of information are in many cases a result of presumption, or an extrapolation from known facts. To understand specific biochemical phenomena at the molecular level, *in vitro* analyses of biochemically characterized samples are strictly required. Conventionally, biochemists had to purify their samples, i.e., proteins and other biomolecules, from living organisms. The recombinant expression technologies that emerged in the late twentieth century helped them greatly. However, although there is a lot of information and a lot of potential targets to be analyzed, the preexisting technologies do not meet the need to prepare sufficient sized samples of many different proteins.

Cell-free protein synthesis was developed in the middle of the last century. It entered the limelight as a method for the preparation of proteins when the continuous-flow cell-free method was developed by Spirin *et al.*[1] in 1988, in which fresh substrates were supplied into and byproducts were removed from the reaction chamber continuously during the reaction with the extract from *E. coli* or wheat embryos. Our group found that the instability that had been observed for the translation with wheat extracts prepared by conventional methods arose from intrinsic factors that catalytically inhibited translation[2], and developed a method to eliminate the catalysts. Here, we describe the method for preparation of the extract as well as the basic techniques for parallel preparation of many different proteins for functional analyses and those for a large-scale preparation that may be applicable to structural analyses and antigen preparation. These technologies are based essentially on the following three elemental technologies: a method for eliminating the contaminants from the endosperm in the wheat embryo extract, which keeps up the inherent robustness of the natural translation apparatus[2], the PCR-based high-throughput method for preparation of DNA templates (the 'split-primer PCR' method)[3] and a reaction format that fit to highly parallel operation (the 'bilayer' method)[4]. A more detailed history of the development of the wheat cell-free system has been reviewed elsewhere[5,6]. The most prominent advantage of the wheat cell-free method, as recognized by our group through the collaborations with many other groups, is the high quality of the produced proteins, particularly when cytosolic proteins from eukaryotic origins are produced[7] (see below in the 'Applications of the method' section).

The cell-free protein synthesis system from *E. coli* is also capable of both highly parallel protein production and mass production. The wheat system has an advantage over the *E. coli* system in the probability of producing human proteins in soluble forms as clearly demonstrated[7]. This is probably due to the eukaryotic nature of the wheat system. In addition, machines for production of proteins are commercially available for the wheat system, as below. On the other hand, the productivity per reaction time may be higher in the *E. coli* system than in the wheat system. In addition, the method for preparation of the cell extract at the laboratory level is less laborious in the *E. coli* system. As a result, the cost of the extract is lower in the *E. coli* system. Thus, if one wishes to produce polypeptides in insoluble forms, the *E. coli* system has a clear advantage both in the cost and the productivity. The *E. coli* system also has an advantage in the production of bacterial soluble proteins that can fold properly in bacterial cells, as the cost of the extract is lower. Although we do not have clear statistical data, it seems that the codon usage bias in the open reading frame (ORF) sequence to be translated affects the productivity much less severely in the wheat system[8].

The wheat cell-free protein synthesis system has been commercialized by CellFree Sciences (CFS). The products of CFS, including the wheat embryo extract (cat. nos. CFS-TRI-1240/1240H/1240G), have highly controlled qualities, and they come with detailed protocols when purchased. Thus, we start with a method for preparation of the extract, for those who are hesitant to purchase the extract. As the extract is stable at −80 °C for years, it is less convenient to prepare small amounts of the extract at a time. In addition, we have already described the small-scale method several times elsewhere[9,10]. Thus, we show here a large-scale method[11]. The extract prepared by this protocol fits to the CFS protocols. We then describe the 'bilayer' methods for small-scale high-throughput parallel protein synthesis in a microtiter plate and those for larger scale preparation, which are essentially the same as that in the CFS protocols and are most convenient at present. A diagram showing the procedure is in **Figure 1**.

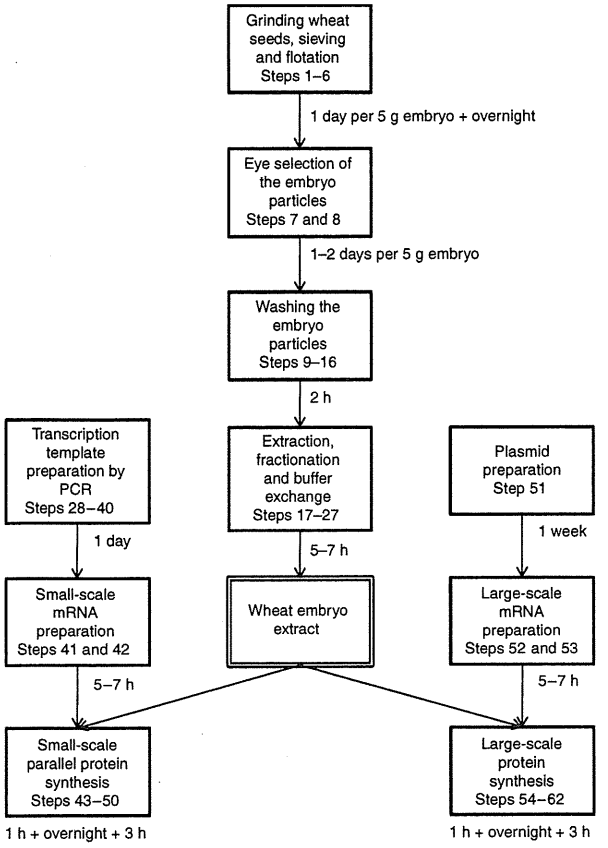

Figure 1 | Summary of steps involved in the procedure.

Experimental design

Preparation of unwashed embryo particles. The primary (unwashed) embryo particles are prepared by crushing wheat seeds with a mill followed by sieving and selection. Typically, 1 kg of wheat seeds gives around 1 g of crude embryo particles.

The most important step in the preparation of a good extract is to obtain an ensemble of good embryo particles that attach minimal amounts of endosperm to be washed out in the next step. This is possible at present only by selecting the particles apparent to human eye. This eye selection is the toughest step for laboratory workers. Thus, we use conventional methods to select the particles crudely by flotation before selection by eye. However, this raises the problem of organic-solvent waste containing carbon tetrachloride. Therefore, we now use the extract supplied by CFS for ordinary experiments. Nevertheless we describe here the method including the flotation selection step, expecting that the researchers who can use carbon tetrachloride in the laboratory may be able to perform the experiments themselves. The flotation steps may be omitted if more time and effort can be invested in the eye selection. We have not tested if other liquids could be used for the purpose, such as pure methylene chloride that has a density close to the one used in the present procedure and high-density aqueous solutions of polymers and/or salts.

Preparation of the extract. The embryo particles should be washed extensively before being crushed. This will eliminate translation inhibitors that come from endosperm. Conventionally, we crushed up to several grams of the washed embryo particles with mortar and pestle under liquid nitrogen in a cold room[2,9,10]. Here, we describe a patented method using a food processing mill/mixer, which has facilitated a larger-scale preparation of the extract with higher activities and has eliminated the risk of choking in the cold room associated with the use of liquid nitrogen[11]. While we used a popular food processor, a conventional Waring Blender can also be used. Although we usually start with 60 g of unwashed embryo particles, the amount of the starting material can be reduced down to several grams. The smallest amount that could be crushed properly is dependent on the size of the blender cup. A machine that can grind green tea may be useful for smaller-scale experiments, although we have not tested any. Once a large amount of the extract is prepared, it can be stored at −80 °C for at least a year.

Conventionally, we prepared the extract in the extraction buffer (EB, see below). However, we found that the buffered substrate mixture as below (BSS) is useful and more convenient than the EB, because preparation of the translation reaction mixture can be simpler, which is important for high-throughput applications.

The translational activator sequences. The mRNA molecules to be translated in the wheat cell-free system should have a translational enhancer sequence in the 5′-untranslated region (UTR)[3,12]. We have observed that the introduction of a cap structure at the 5′ end of mRNA molecules by a standard transcription method does not work well unless the mRNA concentration to be used for translation is optimized for each preparation. The 3′-UTR of the mRNA molecules should be at least as long as 500 nt, while no sequence preferences have been observed. Thus, 3′-UTR can contain the sequence from the vector in which the ORF to be translated is subcloned.

Template preparation by PCR. We describe a method for preparation of transcription-ready PCR fragments with a 5′-enhancer sequence and a long 3′ sequence[3], by which many different cDNA clones could be amplified in parallel. The PCR method comprises two steps. In the first PCR, a 5′ gene-specific primer and the AODA2306 primer (see **Table 1** and **Fig. 2a,b**) are used. The gene-specific primer should be designed for each gene to be expressed so that it hybridizes with the first 20 bases of the ORF to be amplified as in **Table 1**. It should introduce a short leader sequence. The

TABLE 1 | Oligodeoxyribonucleotides.

Name	Sequence	Convenient stock concentration (μM)
AODA2303	5′-GTCAGACCCCGTAGAAAAGA-3′	1
AODA2306	5′-AGCGTCAGACCCCGTAGAAA-3′	0.1
deSP6E01	5′-GGTGACACTATAGAACTCACCTAT CTCCCCAACACCTAATAACATTCAAT CACTCTTTCCACTAACCACCTATCTAC ATCACCACCCACCACCACCAATG-3′	0.1
SPu	5′-GCGTAGCATTTAGGTGACACT-3′	1
Gene-specific primer	5′-CCACCCACCACCACCAATGNNN NNNNNNNNNNNN-3′	0.1

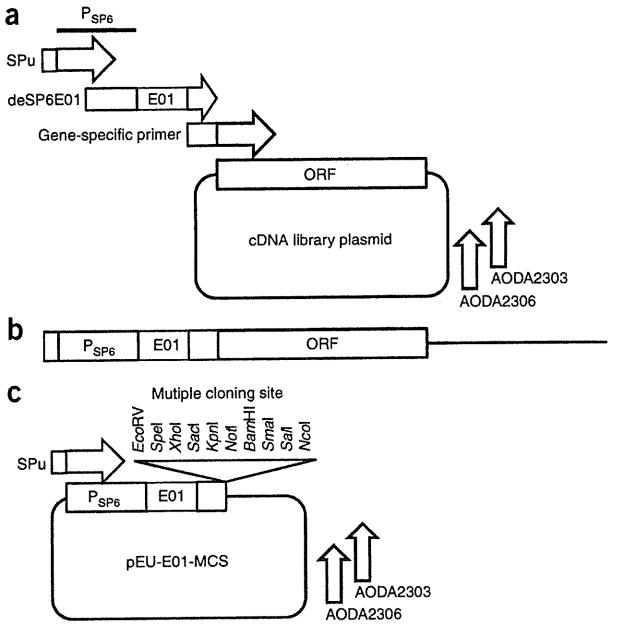

Figure 2 | Schematic representations of DNA molecules. (**a**) Positions of the primers used for the two PCR amplifications are indicated with a schematic representation of a typical cDNA library plasmid clone. The two downstream primers hybridize near the plasmid origin, and thus the library vector should have the same sequence, and the ORF of the library clone should be inserted in this direction. The gene-specific primer and AODA2306 are used for the first PCR, and the other three primers are used for the second PCR. (**b**) An illustration of the transcription-ready template DNA that should be generated after the second PCR. (**c**) An illustration of pEU-E01-MCS, which is provided in the CFS kits and is useful for cloning of the cDNA sequence to be expressed in the wheat cell-free translation system.

AODA2306 primer is designed to hybridize within the replication origin region of the pUC plasmids, which is present in many cDNA library vectors. In the second PCR, AODA2303, deSP6E01 and SPu are used as primers. AODA2303 hybridizes three bases closer to the ORF than the position for AODA2306. deSP6E01 is a long oligonucleotide containing a partial SP6 promoter sequence lacking the 5′ five bases, the E01 sequence, and the leader sequence that is also in the 5′ region of the gene-specific primer. This primer is used in a lower concentration. The E01 5′ enhancer sequence used here can be substituted with the E02 sequence[12] or by the Ω sequence from tobacco mosaic virus. SPu contains the 5′ 14 bases of the SP6 promoter sequence in the 3′ part (the 3′ 9 bases overlaps with the 5′ region of deSP6E01). It is important to split the promoter sequence so that no primer has a complete promoter sequence because this dramatically reduces the possibility of generating nonspecific amplification of transcribable sequences. This 'split-primer' method is not required if the cDNA clones are inserted into pEU as described below.

Small-scale parallel protein synthesis. The PCR products can be transcribed into mRNA by a simple enzymatic reaction. The transcription products can be transferred directly into the translation mixtures. The bilayer method described here is suitable for parallel translation of many different mRNA samples, as it can be performed in microtiter plate wells and is much more efficient than a simple batch reaction. The reaction mixture containing the extract is slightly heavier than the substrate solution, and these two solutions can form a bilayer. Translation starts within a small space with concentrated initiation factors and ribosomes, forming polysomes. As the reaction proceeds, the byproducts are gradually diluted into the upper substrate solution, and the fresh substrates gradually diffuse into the reaction site. Thus, it is very important not to mix the two layers. Robots performing this procedure by parallel operations are available from CFS.

Conventionally, we adjusted the concentrations of the ingredients in the starting reaction mixture that should be layered under the substrate mixture to those of the components in the BSS substrate mixture. However, we have found that this is not necessary, probably because low-molecular-weight compounds will diffuse rapidly into the reaction mixture from the substrate mixture. In addition, it has been found to be unnecessary to remove the white insoluble material generated during the transcription reaction. The protocol shown here is thus quite simple: just mixing three solutions, including the extract, the transcription product and the creatine kinase solution. This simplicity has made this protocol more useful for high-throughput parallel production of many different proteins.

The concentrations of magnesium and potassium ions can affect the translation efficiency, whereas the transcription buffer contains a higher concentration of magnesium ions and no potassium ions, which may cause inefficient translation. However, we have observed no problem in the efficiency of translation. This may be because small ions can exchange rapidly between the reaction and substrate mixtures. It is also possible that the magnesium pyrophosphate precipitate may be serving as a buffer of magnesium concentration.

The pEU expression vector. The pEU vector contains an SP6 promoter, a translational enhancer and a multiple cloning site (**Fig. 2c**). This vector is suitable for large-scale expression of the subcloned ORF in the wheat cell-free system. Control pEU plasmids, such as that harboring the GFP cDNA, are available on request. Various pEU plasmids with inserted tags are also available. We are also ready to distribute a Gateway destination vector of pEU on request: various entry vectors for the human cDNA clones are also available from the National Institute of Technology and Evaluation, Japan[7].

Large-scale protein synthesis. We describe here the method for the bilayer mode large-scale protein synthesis with an mRNA solution prepared by direct transcription of the pEU plasmid template harboring the ORF sequence to be translated. For large-scale synthesis, more productive methods are available (see below). However, the present method is the simplest to be performed and thus fits with automation. The transcription template can also be prepared by PCR amplification of the plasmid sequence with the SPu and AODA2303 primers. We recommend the direct transcription method here just because the method is simpler than that including a PCR step.

Control reactions that should be added. pEU-E01-DHFR, which is available from CFS and from our laboratory, can be used for a positive control reaction for large-scale protein synthesis. For the small-scale experiment, an aliquot from the large-scale transcription product from pEU-E01-DHFR can be used. When only one sample is tested, the sample with no mRNA (water should be

added instead of the mRNA solution) may be used as a negative control, which will help to identify the band of the synthesized protein on the electrophoresis gel among the other bands arising from the components of the extract. If plural different mRNA samples are translated, we usually perform no negative control reaction because each band can be identified by comparison with the other lanes.

Applications of the method

Examples of expressed proteins. We have already tested many different proteins for the productivity in the wheat cell-free system. An encompassing list of successful proteins would be so large that we could not show here. Lists of the successful examples at the time point of 2006 are elsewhere[5]. In such examples, the details of the methods for translation were different from the exact one presented here: as far as we know, success in production of a protein does not depend on the details and the reaction modes described below. Recently, 13,361 human cDNA clones were tested if they were translated in the wheat cell-free system by a bilayer protocol. It was found that 12,996 produced a detectable amount of the polypeptide and that 12,682 were detectable in the soluble fraction, of which 3,040 contain at least one predicted transmembrane domain[7]. Therefore, we believe that most cDNA sequences can be translated into the polypeptide sequences, unless the polypeptide interferes severely with the translation machinery. We have observed that many of the eukaryotic protein kinases can be produced in the active forms in the wheat translation system, while this group of proteins is recognized to be generally difficult to be produced in bacterial systems[5]. In particular, human calcium/calmodulin-dependent protein kinase II delta produced by this method readily phosphorylated novel natural substrates within a HeLa cell extract[13]. We have also produced plant RNA ligase, production of which has been reported to be difficult using bacterial expression systems[14]. It has been suggested that the wheat system has an advantage over bacterial systems in proper folding of multidomain proteins from eukaryotic origins, in particular those with a domain with a high β-strand contents[15]. This may be a result of coevolution of protein sequences and the protein synthesis apparatus, which might have been the origin of various protein functions in eukaryotic cells that confer the dazzling complexity of the eukaryotic organisms. On the other hand, some prokaryote-specific proteins might be produced in an inactive form in the wheat system: we have observed that a bacterial protein with a deep trefoil knot structure is not produced in its fully active form[16]. cDNAs with highly biased codon usages are also difficult to be expressed in bacterial systems. We have already expressed many *Plasmodium* cDNAs that have very high A/T contents successfully[8]. Proteases are generally difficult to be produced *in vitro* because of their activity. However, the wheat cell-free system sustains the translation activity even at 4 °C, and we have already confirmed that some proteases can be produced successfully (our unpublished data). Formation of proper quaternary structures has been observed for several proteins. The crystalline particles of polyhedrin were observed when its mRNA was translated[17]. A heterodimer enzyme was found to fold properly only when synthesized simultaneously in a reaction mixture containing the mRNA molecules for both subunits[18], whereas another heterodimer enzyme had its activity even when each subunit was synthesized separately and mixed with each other after purification[19]. There are only a limited number of examples of disulfide containing proteins and membrane proteins that were expressed efficiently as below. It is very difficult to introduce sugar modifications onto proteins, in part because the endoplasmic reticulum is absent from the extract and, in part, because the extract contains enzymes that degrade sugars.

The PCR method, fusion proteins and high-throughput production. The PCR method presented here can be modified in many ways. In fact, the templates for the human proteins above were amplified from *in vitro* recombinants produced in the Invitrogen Gateway system without transformation[7]. The templates for fusion proteins can be produced easily by PCR, and more than 500 different fusion proteins have been tested for their solubility and activities virtually by one person[15]. This was possible because proteins produced in the wheat system were generally quite stable. The stability is due to the lack of the 26S proteasome-dependent protein degradation activity[20]. It was also possible to manually produce many different proteins with different N-terminal sequences for a systematic analysis of the N-end rule in the wheat cell-free system[21]. N-terminal small tags, such as 6× His tag, can be fused to any protein by simply changing one primer used in the present PCR protocol. Larger tags such as glutathione-*S*-transferase can also be fused by including a small amount of the DNA fragment encoding the tag. These fusion technologies were successfully used for a high-throughput parallel assay of many different transcription factors encoded in cDNA library clones[22]. This type of rapid parallel assay and screening of many different proteins, we believe, will become very important for the post-genomic researches. A basic technology for construction of a protein chip with the proteins fused to a DNA-binding protein has also been developed[23]. The use of PCR for rapid template preparation was also shown to have a potential to accelerate protein engineering[24]. Most of these high-throughput applications have been performed according to older and more complicated protocols than the one that we show here. The present small-scale protein synthesis protocol can be applied to the parallel production of hundreds of different proteins just by performing it in parallel, using a multichannel pipette or the machine mentioned below.

Reaction modes for the cell-free translation. There are several reaction formats for translation. Although we present here the method for translation in the bilayer mode, the other formats are also possible with the extract prepared by the present procedure. Different reaction formats have been summarized elsewhere[9,10]. The batch mode translation, in which the reaction is performed in a homogeneous solution, is useful for testing the activity of the extract and mRNA preparations. For the batch mode synthesis, a fourfold concentrated solution of the substrates (4× BSS, see below) is useful for preparation of the starting mixture in 1× BSS with creatine kinase. In this case, the test can be more sensitive and quantitative if a radiolabeled amino acid is included in the reactions. The dialysis mode translation, in which the reaction is performed within a dialysis bag or a dialysis cartridge with continuous dialysis against the substrate solution, is generally more efficient than the bilayer method. The discontinuous batch (or 'repeat-batch') method[10], in which the buffer/substrate is forcibly exchanged during the translation reaction repeatedly, is very productive, although it needs a machine to be performed. A machine that performs the discontinuous batch mode translation reaction

for gram-scale synthesis is available from CFS, and the protocols for the machine are available in the CFS website. Machines for the bilayer mode parallel translation reactions for high-throughput applications are also available from CFS.

Amino acid labeling for protein structural analyses. The wheat translation system has also been applied to structural biology. This utilizes the high productivity and ease of amino-acid-specific labeling. NMR heteronuclear single quantum coherence (HSQC) spectra could be obtained by measuring the translation product with uniformly labeled amino acids almost without purification: only after a buffer exchange and removal of the precipitant[25]. This is very useful for high-throughput assessing the 'foldedness' of the structural biology samples[26]. The wheat cell-free system has already been modified to fit to a large-scale screening of proteins that are suitable for NMR-based structure determination[26,27]. Amino-acid-specific isotope labeling is also possible with only two transaminase inhibitors added to the translation reaction that prevent scrambling of the isotope caused by metabolic reactions[28–30]. This method may be useful also for the labeling with the stereo-array isotope labeling (SAIL) amino acids, which is expected to accelerate NMR structural analyses[31]. For these applications, the dialysis method may be more useful than the present bilayer method. Selenomethionine substitution of methionine residues in proteins is also easy with the wheat cell-free system, and an X-ray structure of a restriction enzyme has been determined[32].

Modification of the extract. The extract is resistant to many chromatography resins and ultrafiltration membranes. Therefore, it is possible to pretreat the extract with glutathione sepharose or with a metal-chelating resin in order to remove the binders arising from the wheat embryos. Such extracts are available from CFS. It is also possible to freeze-dry the extract without a severe loss of activity, and the freeze-dried extract is stable at least 3 years at −20 °C. We believe that the protein synthesis machinery within embryo cells in natural situations is preserved in a dehydrated state in the winter before imbibition in the spring. We are developing educational experiment kits including a freeze-dried extract, which can be stored stably even in a household freezer that most high schools may be equipped with. It may help the students to feel and understand the relationships between genetic information and protein function and between life and matter.

Other options. Many other applications are possible by modifying the materials added to the reaction. A cofactor-binding protein has been synthesized in the presence and absence of the cofactor, and it was found that both holo-forms and apo-forms could be produced, which was useful for the study of the architecture of the enzyme[10,33]. Disulfide bond formation may be inefficient in the wheat cell-free system because of the presence of DL-dithiothreitol (DTT) in the reaction mixture. By omitting DTT from the substrate mixture, disulfide bonds in some proteins may be formed, although the efficiency of protein synthesis are limited[7,34]. Some membrane proteins have been synthesized in their active forms in the presence of liposomes or some detergents[35–37]. It was also possible to select some functional sequences from a random pool of mRNA molecules, through which the E01 sequence used in the present protocols has been obtained[12]. The lack of the proteasome activity[20] may help us to reconstitute intracellular multicomponent molecular systems, such as protein degradation systems, without purification of each component. We believe that the wheat translation system may be useful also for synthetic biology purposes.

MATERIALS

REAGENTS

- Unsterilized wheat seeds (strain 'Chihoku': any strain may be used): dried after harvesting, unbaked and containing no pesticides or insecticides (as one may be exposed to the drugs when crushing the seeds)
- Nonidet P-40 (NP-40; Nacalai Tesque, cat. no. 23640-94) ! **CAUTION** Harmful (wear gloves).
- Cyclohexane (Wako Pure Chemicals, cat. no. 034-05001) ! **CAUTION** Highly flammable, harmful and dangerous for the environment (wear gloves and handle the reagent in a fume hood).
- Carbon tetrachloride (Wako Pure Chemicals, cat. no. 039-01271) ! **CAUTION** Toxic and dangerous for the environment. Use of this reagent is tightly regulated in Japan. Wear gloves and handle the reagent in a fume hood. Confirm and obey local regulations associated with the use and disposal of the reagent.
- 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; Nacalai Tesque, cat. no. 17514-15) ! **CAUTION** Irritant.
- Potassium hydroxide (Nacalai Tesque, cat. no. 28616-45) ! **CAUTION** Corrosive (wear gloves and protecting glasses).
- Potassium acetate (Nacalai Tesque, cat. no. 28405-05)
- Magnesium acetate tetrahydrate (Nacalai Tesque, cat. no. 20821-85)
- Calcium chloride (Nacalai Tesque, cat. no. 06729-55)
- DL-Dithiothreitol (DTT; Wako Pure Chemicals, cat. no. 049-08972)
- Standard 20 L-amino acids (Wako Pure Chemicals or Nacalai Tesque)
- Milli-Q water (freshly prepared with a Millipore system, Millipore)
- Elix water (produced with a Millipore system, Millipore)
- Sodium acetate (Nacalai Tesque, cat. no. 31119-65)
- Acetic acid (Nacalai Tesque, cat. no. 00212-56) ! **CAUTION** Flammable (handle in a fume hood).
- Ethanol (Nacalai Tesque, cat. no. 14713-95) ! **CAUTION** Highly flammable (handle in a fume hood).
- Sephadex G-25 Fine (GE Healthcare, cat. no. 17-0032-01)
- TaKaRa *Ex* Taq (Takara Bio, cat. no. RR001A)
- Plasmid preparation kit (QIAGEN Plasmid Midi Kit, QIAGEN, cat. no. 12143)
- ATP, disodium salt (Sigma, cat. no. A3377)
- GTP, sodium salt (Sigma, cat. no. G8877)
- CTP, disodium salt (Sigma, cat. no. C1506)
- UTP, trisodium salt (Sigma, cat. no. U6625)
- Spermidine (Rnase-free, Sigma, cat. no. S0266) ! **CAUTION** Corrosive (wear gloves).
- Creatine phosphate (Wako Pure Chemicals, cat. no. 030-04584, or Roche, cat. no. 621722)
- Creatine kinase (Roche, cat. no. 127566)
- SP6 RNA polymerase (HC) (80 U μl^{-1}, Promega, cat. no. P4084)
- RNasin Ribonuclease Inhibitor (20–40 U μl^{-1}, Promega, cat. no. N2511)
- Oligodeoxyribonucleotides listed in **Table 1** (Invitrogen)
- Liquid nitrogen ! **CAUTION** Wear nonpermissible gloves; obey local regulations.

EQUIPMENT

- Rotor Speed Mill PULVERISETTE 14 (Fritsch)
- Sieve shaker (A-3 PRO, Fritsch) with 710-, 850- and 1,000-μm mesh sieves (The Iida Testing Sieves, Iida Manufacturing)
- Sonicator (W-113 Ultrasonic Cleaner, Honda Electronics)
- Fume hood
- Blender (KC-4811W Mill & Mixer, Twinbird)
- Amicon Ultra-15 (10 kDa, Millipore, cat. no. UFC9 010 08)
- Toothpicks
- Mesh skimmer
- Kimwipe sheets
- Kim Towels
- Corner trash bags ('Gomipon', Kokubo): alternatively, nylon stockings may be useful

- Hitachi CR21G centrifuge with an R10A3 rotor (Hitachi, cat. no. 904308D0) with 500-ml centrifuge bottles (cat. no. 330437A)
- TOMY GRX-220 centrifuge with a TA-24BH rotor (TOMY), with 50-ml round-bottom centrifuge tubes (cat. no. 3177-9500; capped with DS3119-0029 centrifuge tube closures)
- 50-ml injection syringe (Terumo, cat. no. SS-50ESZ)
- Cotton wool
- Aluminum foil
- Air incubator (MIR-153, SANYO)
- UV spectrophotometer (Beckman DU600, Beckman)
- Submarine-type and slab gel electrophoresis systems for agarose and SDS-polyacrylamide gel electrophoresis, respectively
- UV light and camera (Toyobo)
- PCR machine (PCR Thermal Cycler MP, Takara Bio)
- 96-well microtiter plate (Techno Plastic Products AG, cat. no. 92096)
- 6-well plate (Techno Plastic Products AG, cat. no. 92006)
- pH meter
- Autoclave

REAGENT SETUP

Cyclohexane/carbon tetrachloride mixture Mix 2.4 volume of carbon tetrachloride with one volume of cyclohexane. Handle and store it in a fume hood at room temperature (20–25 °C). The mixture can be reused several times.

0.5% (vol/vol) NP-40 solution Dissolve NP-40 in Milli-Q water. Store it at room temperature (stable for years).

3 mM amino acid mixture Dissolve all of the 20 standard amino acids into Milli-Q water. For storage, seal the container tightly to avoid air oxidation and store it frozen at −20 °C (stable for at least a year).

2× EB Mix 80 mM HEPES-KOH (pH 7.6), 200 mM potassium acetate, 10 mM magnesium acetate, 4 mM calcium chloride, 0.6 mM amino acids and 8 mM DTT freshly at 4 °C. ▲ **CRITICAL** Use it in a few days.

5× Transcription buffer (TB) Mix 400 mM HEPES-KOH (pH 7.8), 80 mM magnesium acetate, 10 mM Spermidine and 50 mM DTT. Store it in aliquots at −20 °C (stable for at least a year).

3 M Sodium acetate Add 3 M acetic acid to a 3 M sodium acetate solution and adjust to pH 5.2. Sterilize the solution by autoclaving or by filtration. Store it at room temperature (stable at least a year).

70% (vol/vol) ethanol Mix 35 ml of ethanol and 15 ml of Milli-Q water. Store at −20 °C (stable at least a year).

100 mM ATP, CTP, GTP and UTP Dissolve the powder of the salt of the nucleotide in water and adjust pH of the solution between 7 and 8.5. Measure the absorbance at 260 nm and adjust the concentration to 100 mM by adding water according to the molecular extinction coefficient of 15.4, 9.0, 11.4 and 9.9×10^3 cm^{-1} M^{-1} for ATP, CTP, GTP and UTP, respectively. Store each solution frozen at −80 °C (stable for a year).

NTP solution Mix equal volume of 100 mM ATP, CTP, GTP and UTP. Store it frozen at −80 °C (stable for a year).

20 mg ml^{-1} Creatine kinase Dissolve the powder in water and store it in aliquots at −80 °C (stable at least a year).

4× Buffered substrate solution (BSS) 120 mM HEPES-KOH (pH 7.6), 400 mM potassium acetate, 10.8 mM magnesium acetate, 1.6 mM spermidine, 10 mM DTT, 1.2 mM amino acids, 4.8 mM ATP, 1 mM GTP and 64 mM creatine phosphate. Store it in aliquots at −20 °C (stable for 2 months).

1× BSS Dilute 4× BSS with Milli-Q water. Prepare just before use; can be stored for a few days at −20 °C.

EQUIPMENT SETUP

Rotor speed mill Set the rotor speed to 7,000 r.p.m. The mill and the sieve shaker will produce a lot of fine powder of flour, which floats around in the air and contains inhibitors of protein synthesis. Thus, these equipments may be better kept away or spatially isolated from the biochemical laboratory.

A-3 PRO shaker Set the amplitude to 2.0 mm, sieving time to 2 min and interval to 1 s. This may also be isolated from the biochemistry laboratory.

The blender Use the larger cup (200 ml mixer cup) for 60 g embryo. The smaller (70 ml) cup may be useful for the preparation from smaller amounts (less than around 20 g) of the purified embryo particles.

Sonicator Add ice to the water in the top bucket. Set the frequency to 45 kHz.

PCR thermal cycler Program 1: 94 °C for 4 min; 30 cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 1 min per 1-kb amplified sequence; and 72 °C for 5 min. Program 2: 94 °C for 4 min; 5 cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 1 min per 1-kb amplified sequence; 35 cycles of 98 °C for 10 s, 60 °C for 40 s and 72 °C for 1 min per 1-kb amplified sequence; and 72 °C for 7 min.

PROCEDURE

Preparation of unwashed embryo particles ● TIMING 2–3 d per 5 g embryo particles from 5 to 6 kg seeds

1| Grind the wheat seeds in the mill at the rate of 100 g per min. Repeat this four times.

! **CAUTION** Wear protectors if needed, because fine powder will drift around in the air.

2| Shake the sample in the sieve shaker.

3| Collect the particles on the 850- and 710-μm sieves in a dish.

4| Let the sample fall onto another dish from around a 50-cm height repeatedly to remove seed-coat fragments.

5| Pour the particles into a beaker containing around 1 l of cyclohexane/carbon tetrachloride mixture in the fume hood and stir the mixture thoroughly. Do not leave the embryo particles in the solvent too long.

6| Collect the floating particles with a mesh skimmer as fast as possible after the particles are separated, and put them on Kimwipe sheets in the fume hood to remove the solvent overnight.

■ **PAUSE POINT** The embryo particles can be stored at 4 °C for several years.

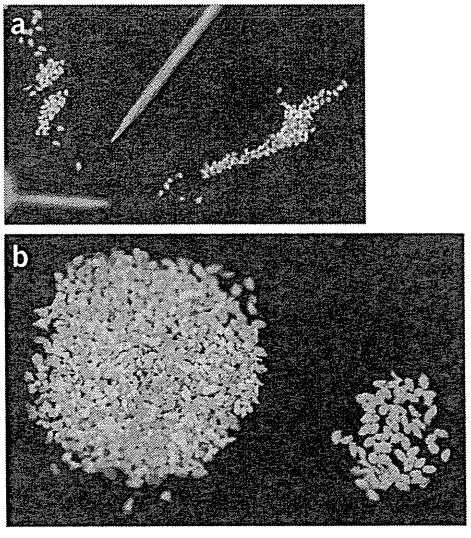

Figure 3 | Selection of good embryo particles by eye. (**a**) Selecting good particles using a toothpick. (**b**) Good particles (left) and bad particles (right).

7| Spread the particles on a clean paper or plastic sheet on a desk. Select only those particles with yellow color with a minimum amount of white matter attached to them using a toothpick, carefully investigating each particle by eyes: remove brownish particles and the particles with much white matter coming from endosperm (**Fig. 3**).
▲ **CRITICAL STEP** The white matter contains the catalytic inhibitors of protein synthesis.

8| Store the selected particles at 4 °C until use.
■ **PAUSE POINT** The embryo particles can be stored at 4 °C for several years.

Preparation of the extract ● TIMING **1 d**

9| Put 60 g of embryos in a corner trash bag. A smaller amount may also be washed successfully, although we have only a little experience. Wash the particles in a stream of 5-l cold Elix water.

10| Dip the bag in cold Elix water (4 °C, typically 700 ml) and knead it gently. Do not knead it too much.

11| Change the water and repeat Step 10 several times more until no white matter disperses out through the bag.

12| Change the water to 500 ml of 0.5% NP-40 solution (4 °C) and sonicate the sample for 5 min with gentle stirring.

13| Wash in an Elix water stream (typically 3 liters, not chilled) until no bubbles can be seen, and sonicate twice in cold Elix water.

14| Wash the particles five times more in a beaker with 800 ml each of cold Milli-Q water.

15| Take out the embryo particles and wrap them in Kimwipe sheets, which are further wrapped with a sheet of Kim Towel, in order to remove water.

16| Repeat this wiping a few times until no more water can be removed.

17| Put the washed embryo particles from 60 g of the starting unwashed embryos (around 120 g) in the larger (mixer) cup of the blender and add 90–120 ml of 2× EB (4 °C). We have confirmed that 10 g of the washed embryo particles could also be processed successfully with 10 ml of 2× EB in the smaller cup.

18| Run the blender for 30 s three times.

19| Centrifuge the sample at 30,000*g* at 4 °C for 30 min in a TA-24BH rotor with the GRX-220 centrifuge (**Fig. 4**). Collect the supernatant, i.e., the middle layer between the fatty material and the precipitate.

20| Centrifuge the sample again at 30,000*g* at 4 °C for 15 min in a TA-24BH rotor with the GRX-220 centrifuge and collect the supernatant in a tube.

21| Pass the sample through a G-25 column pre-equilibrated with 1× EB. A 40-ml column may be prepared in a 50-ml injection syringe with cotton wool at the bottom, which can be hung at the rim of a 500-ml centrifuge bottle in it, loaded with maximum of 20 ml of the sample, capped with aluminum foil and centrifuged at 750*g* for 5 min at 4 °C in a R10A3 rotor.

22| Pass the sample through a G-25 column pre-equilibrated with 1× BSS.

23| Measure the absorbance of the extract at 260 nm in a 1-cm path length cuvette (a several hundred-fold dilution will be needed), which may be more than 150. If the absorbance is, e.g., 160, then the concentration of the extract is 160 AU ml^{-1}, where '1 AU' is the amount of the extract that gives the absorbance of 1 at 260 nm in a 1-cm

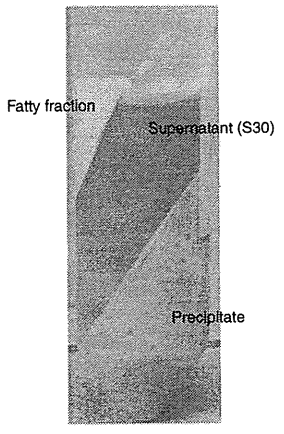

Figure 4 | An example of the sample after the first 30,000*g* centrifugation. The sample in Step 19 may be separated as in the figure (in which a conical tube is used).

path if dissolved in a 1-ml solution. The manuals from CFS use 'OD' instead of 'AU.'
? TROUBLESHOOTING

24| Concentrate the extract to around 300 AU ml^{-1} by ultrafiltration with 10 kDa Amicon Ultra-15 cartridges. Eight cartridges can be centrifuged at once in the TA-24BH rotor.

25| Pass the sample through a G-25 column pre-equilibrated with 1× BSS again.

26| Measure the absorbance at 260 nm of the extract and adjust the concentration to 240 AU ml^{-1}.
? TROUBLESHOOTING

27| Aliquot the sample and freeze each tube quickly in liquid nitrogen. Store them at −80 °C. Do not store the extract at −20 °C.
■ **PAUSE POINT** The extract is stable for at least a few years at −80 °C.

Template DNA preparation for small-scale parallel protein synthesis ● TIMING 1 d
28| Mix 0.5 ng of the plasmid containing the target cDNA sequence in a PCR tube on ice with 10 nM of the target-specific primer, 10 nM of the AODA2306 primer, 200 μM each of dNTP, the buffer supplied by the supplier and 0.0125 U μl^{-1} of *Ex* Taq DNA polymerase in a 20-μl reaction mixture (**Fig. 2**). When many different reactions are performed in parallel, mix the materials other than the plasmid and the target-specific primer in one tube and dispense this mixture to the plasmid/target-specific primer solution. Set the sample(s) in PCR Thermocycler and run it with Program 1.

29| Check the amplified DNA by agarose gel electrophoresis.
■ **PAUSE POINT** PCR products may be stable at −20 °C for years.
? TROUBLESHOOTING

30| Mix 5 μl of the first PCR product (without any purification) in a 200-μl PCR tube with 100 nM each of the SPu and AODA2303 primers, 1 nM of the deSP6E01 primer, 200 μM each of dNTP, the buffer supplied by the supplier and 0.0125 U μl^{-1} of *Ex* Taq DNA polymerase in a 50-μl reaction mixture (**Fig. 2**). For parallel preparation, mix everything other than the first PCR product in one tube and dispense it into each PCR tube before putting in the first PCR product. Put the sample(s) in PCR Thermocycler and run it with Program 2.

31| Add 5 μl of 3 M sodium acetate (pH 5.2) and 140 μl of ethanol to each of the second PCR solutions. Mix the samples well. Incubate them at −20 °C for 10 min.

32| Centrifuge the samples at 15,000*g* for 15 min at 4 °C in a standard microcentrifuge.

33| Remove the supernatant and add 300 μl of chilled 70% ethanol.

34| Centrifuge the samples at 15,000*g* for 5 min at 4 °C.

35| Remove the supernatant carefully and thoroughly and dry the samples with the tube lids open covered with Saran Wrap.

36| Add 10 μl of Milli-Q water to each sample and dissolve the pellets well.

37| Analyze 1 μl of the samples by agarose gel electrophoresis. Estimate the concentration of the DNA by comparing with the bands of the molecular weight marker run in the same gel. Adjust the concentration of each sample around 0.25 $\mu g\ \mu l^{-1}$.
■ **PAUSE POINT** PCR products are stable at −20 °C for years.
? TROUBLESHOOTING

mRNA preparation for small-scale parallel protein synthesis ● TIMING 5–7 h
38| Prepare a solution containing 6 μl of 5× TB, 3 μl of 25 mM NTP, 48 U of RNase inhibitor and 48 U of SP6 RNA polymerase per 16 μl using Milli-Q water. This gives the 1.5× final buffer concentration. Dispense 16 μl of this solution in each well of a microtiter plate. Add 4 μl of the second PCR solution and incubate the plate at 37 °C for 4–6 h. White insoluble material will be generated during transcription.

39| Analyze the product (1 μl) in a 1% (wt/vol) agarose gel stained with ethidium bromide.
■ **PAUSE POINT** The sample can be stored at −80 °C for weeks. Transportation with dry ice is not recommended.
? TROUBLESHOOTING

Small-scale parallel protein synthesis ● TIMING **1 h + an overnight reaction + 3 h**

40| Keep the mRNA tubes in room temperature. Thaw the extract in water and put it on ice immediately after it has thawed. Thaw the creatine kinase solution on ice. Thaw 1× BSS on ice and mix it well. Spin down the thawed solutions.

41| Dispense 10 μl of 240 OD per ml extract and 0.8 μl of 1 mg ml^{-1} creatine kinase into each well of a microtiter plate on ice. This microtiter plate can be replaced with standard microtubes or PCR tubes if the number of the samples is small.

42| Resuspend the mRNA solutions, which contain white insoluble material, and transfer 10 μl of each suspension into each well of the microtiter plate. Mix the samples gently by pipetting, avoiding bubbles.

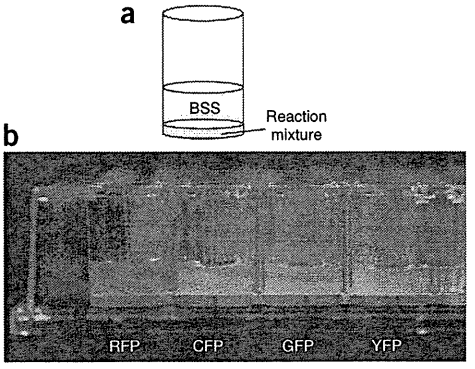

Figure 5 | A schematic representation of the bilayer to be formed at the start of the translation reaction (**a**) and a typical result of translation (**b**). (**a**) In the small-scale protein synthesis, the reaction mixture is layered under the buffered substrate solution (BSS). The two solutions will mix together gradually during incubation. (**b**) Fluorescent proteins synthesized in microtiter plate wells.

43| Dispense 206 μl of 1× BSS in each well of a flat-bottomed microtiter plate.

44| Take each of the mRNA/extract mixtures into a micropipette tip so that no air is at the end of the tip. Insert the tip at the bottom of a microtiter well containing 1× BSS carefully with holding the mixture within the tip and then carefully pump out the mixture under the buffer without mixing, avoiding bubbles, so that the mRNA/extract mixture and the buffer form a bilayer (**Fig. 5a**). Do not mix the samples.

▲ **CRITICAL STEP** Do not mix the samples. It is very important at the start of the reaction that the starting reaction mixture forms a distinct layer that forms a clear boundary with the upper BSS liquid.

45| Seal the wells to avoid evaporation. Be careful not to shake the plate too much.

46| Leave the plate in the air incubator at 15 °C for 20 h.

47| Analyze the sample (3–5 μl) on a standard SDS gel.

? TROUBLESHOOTING

Transcription of a pEU plasmid harboring a target ORF sequence ● TIMING **5–7 h excepting Step 48**

48| Subclone the target ORF into pEU (**Fig. 2c**) and prepare the plasmid using a standard plasmid preparation kit, such as QIAGEN Plasmid Midi Kit. Dissolve the plasmid in the standard TE buffer. Determine the concentration and purity of the DNA sample by measuring the absorbance values at 260 and 280 nm. If the A_{260}/A_{280} ratio is not between 1.70 and 1.85, then further purify the sample by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation with rinsing the pellet with 70% ethanol. Adjust the concentration to 1 μg μl^{-1} with TE.

▲ **CRITICAL STEP** It is recommended to always perform the appended purification steps, because most plasmid preparation kits use an RNase, and because even a small amount of RNase would inhibit the transcription and translation.

■ **PAUSE POINT** Plasmids can be stored for years at −20 °C.

49| Mix 25 μg of plasmid DNA in 250 μl of the transcription buffer containing 50 μl of 5× TB, 25 μl of 25 mM NTP, 250 U of RNase inhibitor and 250 U of SP6 RNA polymerase and incubate this mixture at 37 °C for 6 h. White insoluble material will be generated during transcription. Perform the control reaction with pEU-E01-DHFR or pEU-E01-GFP.

50| Check the sample in an agarose gel. Transcription stops partially at the plasmid replication origin.

■ **PAUSE POINT** The transcription product can be stored at −80 °C for several weeks. Transportation with dry ice is not recommended.

? TROUBLESHOOTING

Large-scale protein synthesis ● TIMING **1 h + an overnight reaction + 3 h**

51| Put the mRNA tubes in room temperature. Thaw the extract in water and put it on ice immediately after it has thawed. Thaw the creatine kinase 20 mg ml^{-1} solution on ice. Thaw 1× BSS on ice and mix it well. Spin down the thawed solutions.

52| Dispense 250 µl of 240 OD per ml extract and 1 µl of 20 mg ml^{-1} creatine kinase into each fresh microtubes on ice.

53| Resuspend the mRNA solutions, which contain white insoluble material, and transfer 250 µl of each suspension into each microtube containing the extract and creatine kinase. Mix the samples gently by pipetting, avoiding bubbles.

54| Dispense 5.5 ml of 1× BSS in each well of a flat-bottomed six-well plate.

55| Take each of the mRNA/extract mixtures into a micropipette tip so that no air is at the end of the tip. Insert the tip at the bottom of a microtiter well containing 1× BSS carefully by holding the mixture within the tip and then carefully pump out the mixture under the buffer without mixing, avoiding bubbles, so that the mRNA/extract mixture and the buffer form a bilayer. Do not mix the samples. Do not shake the plate.
▲ **CRITICAL STEP** Do not mix the samples. It is very important at the start of the reaction that the starting reaction mixture forms a distinct layer that forms a clear boundary with the upper BSS liquid.

56| Seal the plate to avoid evaporation. Be careful not to shake the plate too much.

57| Leave the plate in the air incubator at 15 °C for 20 h.

58| After the incubation, mix the samples for further analyses.

59| To check the products, load 3 µl of the samples on a standard SDS gel.
? TROUBLESHOOTING

● TIMING
Steps 1–8, preparation of unwashed embryo particles: 2–3 d per 5 g embryo particles from 5 to 6 kg seeds
Steps 9–27, preparation of the extract: 1 d
Steps 28–37, template DNA preparation for small-scale parallel protein synthesis: 1 day
Steps 38 and 39, mRNA preparation for small-scale parallel protein synthesis: 5–7 h
Steps 40–47, small-scale parallel protein synthesis: 1 h + an overnight reaction (20 h) + 3 h
Step 48–50, transcription of a pEU plasmid harboring a target ORF sequence: 5–7 h excepting Step 48
Steps 51–59, large-scale protein synthesis: 1 h + an overnight reaction (20 h) + 3 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2.** In our experience, most of the troubles come from a problem during the construction of the DNA molecules that are used for PCR and/or transcription.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
23	Low absorbance	Grinding was not sufficient	This could be foreseen by the color of the supernatant in Step 19
26	Absorbance lower than 240	Insufficient condensation in Step 24	Concentrate the sample again, or leave it as it is
29	No band	Bad template	Check the template cDNA. The plasmid should have the pUC origin and the cDNA sequence
		The specific primer does not hybridize well	Lower the annealing temperature of the PCR program. Extend the target region of the primer
		Primer-dimer involving the specific primer	Extend the target region of the primer
	Nonspecific bands	Hybridization of AODA2306 within the ORF	Try proceeding to Steps 30–37. If the result is still bad, try another primer that hybridizes near the replication origin. For screening purposes, judge if one wishes to stick to this sample
37	No band	Loss of the pellet in the Step 33 or 35	Restart from Step 30
	Nonspecific bands	Nonspecific hybridization within the ORF	Try translation if the main band is correct. For screening purposes, judge if one wishes to stick to this sample

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
39	No band	dNTP instead of NTP added to the reaction	Try again being careful not to confuse NTP with dNTP
	Ladder in the high mobility region	Contamination by Rnases	Extract the template DNA with phenol/chloroform
	Unexpected low mobility extra bands	Efficient transcription	This usually causes no problem in translation
	Very bright bands	Sample is not denatured and is complexed with Spermidine	Denature the sample in a formamide loading dye, which may be the one used for denaturing gels, before applying to the gel
47 or 59	No product	Bad mRNA	Check the mRNA and template DNA again
	No or very faint band	Inefficient translation	Try the small-scale translation again with ^{14}C-labeled leucine added to the reaction and BSS and detect the product by autoradiography or by counting the radioactivity in the acid-insoluble fraction of the reaction mixture
		Alternative translation initiation (out of frame)	Eliminate Gs from between the E01 enhancer sequence and the initiation codon
	Doublet band	Alternative translation initiation (in frame)	Eliminate Gs from between the E01 enhancer sequence and the initiation codon
50	Smear or ladder in the high-mobility region	Contamination by RNase	Further purify the plasmid template as in Step 47
	No band	dNTP instead of NTP added to the reaction	Try again being careful not to confuse NTP with dNTP
		Bad plasmid	Check the plasmid DNA
59	No band	Bad plasmid	Check the plasmid DNA
	Unexpected bands in the low-mobility region	Post-translational modification	Post-translational modification may occur for some proteins We have no unified methodology

ANTICIPATED RESULTS

We show here a typical result of small-scale bilayer mode synthesis of fluorescent proteins (**Fig. 5b**). An SDS gel showing a typical result of synthesis of dihydrofolate reductase (DHFR) is in **Figure 6**. It is difficult to show an averaged amount of produced protein per 1-ml reaction because we do not have a reliable statistic data with the protocol shown here and because the productivity per reaction volume including BSS can vary with the relative volume of BSS to the reaction mixture. However, the average yield per

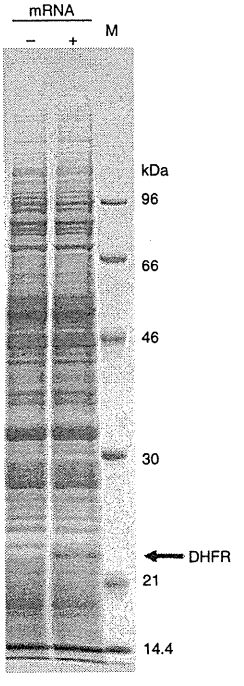

Figure 6 | A typical result of the small-scale bilayer synthesis of DHFR. DHFR was synthesized by the bilayer method using the CFS extract (CFS-TRI-1240), and a 3-μl aliquot of the mixed sample was separated on an SDS gel stained with CBB (Step 47). The left lane (mRNA+), a control reaction product with no mRNA added; the center lane (mRNA–), the product with the DHFR mRNA; and the right lane (M), marker proteins with molecular masses indicated on the right. The amount of DHFR synthesized was 80 ng μl^{-1} (1.8 mg per 1-ml extract). In the parallel protein synthesis experiments, the 'mRNA–' reaction can usually be omitted because different samples can serve as the markers indicating the positions of the bands of the wheat embryo proteins. Modified from a figure kindly provided by R. Morishita, CellFree Sciences.

1-ml extract may be around 0.3 mg both in the small- and large-scale bilayer method. In fact, the average amount per a 150-μl reaction containing 12 μl of the extract for the 13,000 different human ORFs was 4.2 μg, which means 0.35 mg per 1-ml extract[7].

ACKNOWLEDGMENTS This work was supported in part by the Special Coordination Funds for Promoting Science and Technology (Y.E.) and in part by a Grant-in-Aid for Scientific Research on the Priority Areas (No. 20034040 to K.T.) by the Ministry of Education, Culture, Sports, Science and Technology, Japan. We are grateful to Dr. R. Morishita and Mr. Y. Tanaka of CFS for providing pictures and for checking the paper.

AUTHOR CONTRIBUTIONS K.T. collected information and wrote the paper; T.S. prepared the data and pictures; and Y.E. supervised the study.

Published online at http://www.natureprotocols.com/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

1. Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y. & Alakhov, Y.B. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242**, 1162–1164 (1988).
2. Madin, K., Sawasaki, T., Ogasawara, T. & Endo, Y. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA* **97**, 559–564 (2000).
3. Sawasaki, T., Ogasawara, T., Morishita, R. & Endo, Y. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA* **99**, 14652–14657 (2002).
4. Sawasaki, T. *et al.* A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.* **514**, 102–105 (2002).
5. Endo, Y. & Sawasaki, T. Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.* **17**, 373–380 (2006).
6. Takai, K., Sawasaki, T. & Endo, Y. Development of key technologies for high-throughput cell-free protein production with the extract from wheat embryos. *Adv. Protein Chem. Struct. Biol.* **75**, 53–84 (2008).
7. Goshima, N. *et al.* Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome. *Nat. Methods* **5**, 1011–1017 (2008).
8. Tsuboi, T. *et al.* Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect. Immun.* **76**, 1702–1708 (2008).
9. Sawasaki, T. *et al.* The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information. *Methods Mol. Biol.* **310**, 131–144 (2005).
10. Sawasaki, T. & Endo, Y. Chapter 6. Protein expression in the wheat-germ cell-free system. In *Expression Systems* (eds. Dyson, M.R. & Durocher, Y.) 87–108 (Scion Publishing Ltd., Oxfordshire, Oxford, UK, 2007).
11. Endo, Y., Dohi, N. & Nakagawa, M. Germ extract for cell-free protein synthesis and process for producing the same. International Patent WO/2003/064671 filed 31 January 2003.
12. Kamura, N., Sawasaki, T., Kasahara, Y., Takai, K. & Endo, Y. Selection of 5′-untranslated sequences that enhance initiation of translation in a cell-free protein synthesis system from wheat embryos. *Bioorg. Med. Chem. Lett.* **15**, 5402–5406 (2005).
13. Masaoka, T., Nishi, M., Ryo, A., Endo, Y. & Sawasaki, T. The wheat germ cell-free based screening of protein substrates of calcium/calmodulin-dependent protein kinase II delta. *FEBS Lett.* **582**, 1795–1801 (2008).
14. Makino, S., Sawasaki, T., Tozawa, Y., Endo, Y. & Takai, K. Covalent circularization of exogenous RNA during incubation with a wheat embryo cell extract. *Biochem. Biophys. Res. Commun.* **347**, 1080–1087 (2006).
15. Hirano, N., Sawasaki, T., Tozawa, Y., Endo, Y. & Takai, K. Tolerance for random recombination of domains in prokaryotic and eukaryotic translation systems: limited interdomain misfolding in a eukaryotic translation system. *Proteins* **64**, 343–354 (2006).
16. Nureki, O. *et al.* Deep knot structure for construction of active site and cofactor binding site of tRNA modification enzyme. *Structure* **12**, 593–602 (2004).
17. Ikeda, K. *et al.* Immobilization of diverse foreign proteins in viral polyhedra and potential application for protein microarrays. *Proteomics* **6**, 54–66 (2006).
18. Matsumoto, K. *et al.* Production of yeast tRNA (m^7G46) methyltransferase (Trm8-Trm82 complex) in a wheat germ cell-free translation system. *J. Biotechnol.* **133**, 453–460 (2008).
19. Kanno, T., Kasai, K., Ikejiri-Kanno, Y., Wakasa, K. & Tozawa, Y. *In vitro* reconstitution of rice anthranilate synthase: distinct functional properties of the alpha subunits OASA1 and OASA2. *Plant Mol. Biol.* **54**, 11–22 (2004).
20. Takahashi, H., Nozawa, A., Seki, M., Shinozaki, K., Endo, Y. & Sawasaki, T. A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis. *BMC Plant Biol.* **9**, 39 (2009).
21. Kanno, T. *et al.* Sequence specificity and efficiency of protein N-terminal methionine elimination in wheat-embryo cell-free system. *Protein Expr. Purif.* **52**, 59–65 (2007).
22. Kobayashi, T., Kodani, Y., Nozawa, A., Endo, Y. & Sawasaki, T. DNA-binding profiling of human hormone nuclear receptors via fluorescence correlation spectroscopy in a cell-free system. *FEBS Lett.* **582**, 2737–2744 (2008).
23. Sawasaki, T. *et al.* Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. *FEBS Lett.* **582**, 221–228 (2008).
24. Kanno, T. *et al.* Structure-based *in vitro* engineering of the anthranilate synthase, a metabolic key enzyme in the plant tryptophan pathway. *Plant Physiol.* **138**, 2260–2268 (2005).
25. Morita, E.H., Sawasaki, T., Tanaka, R., Endo, Y. & Kohno, T. A wheat germ cell-free system is a novel way to screen protein folding and function. *Protein Sci.* **12**, 1216–1221 (2003).
26. Vinarov, D.A. *et al.* Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nat. Methods* **1**, 149–153 (2004).
27. Vinarov, D.A., Loushin Newman, C.L. & Markley, J.L. Wheat germ cell-free platform for eukaryotic protein production. *FEBS J.* **273**, 4160–4169 (2006).
28. Morita, E.H. *et al.* A novel way of amino acid-specific assignment in ^{1}H-^{15}N HSQC spectra with a wheat germ cell-free protein synthesis system. *J. Biomol. NMR* **30**, 37–45 (2004).
29. Kohno, T. Production of proteins for NMR studies using the wheat germ cell-free system. *Methods Mol. Biol.* **310**, 169–185 (2005).
30. Kohno, T. & Endo, Y. Production of protein for nuclear magnetic resonance study using the wheat germ cell-free system. *Methods Mol. Biol.* **375**, 257–272 (2007).
31. Kainosho, M. *et al.* Optimal isotope labelling for NMR protein structure determinations. *Nature* **440**, 52–57 (2006).
32. Miyazono, K. *et al.* Novel protein fold discovered in the PabI family of restriction enzymes. *Nucleic Acids Res.* **35**, 1908–1918 (2007).
33. Abe, M. *et al.* Detection of structural changes in a cofactor binding protein by using a wheat germ cell-free protein synthesis system coupled with unnatural amino acid probing. *Proteins* **67**, 643–652 (2007).
34. Kawasaki, T., Gouda, M.D., Sawasaki, T., Takai, K. & Endo, Y. Efficient synthesis of a disulfide-containing protein through a batch cell-free system from wheat germ. *Eur. J. Biochem.* **270**, 4780–4786 (2003).
35. Nozawa, A. *et al.* A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters. *Plant Cell Physiol.* **48**, 1815–1820 (2007).
36. Goren, M.A. & Fox, B.G. Wheat germ cell-free translation, purification, and assembly of a functional human stearoyl-CoA desaturase complex. *Protein Expr. Purif.* **62**, 171–178 (2008).
37. Kaiser, L., Graveland-Bikker, J., Steuerwald, D., Vanberghem, M., Herlihy, K. & Zhang, S. Efficient cell-free production of olfactory receptors: detergent optimization, structure, and ligand binding analyses. *Proc. Natl. Acad. Sci. USA* **105**, 15726–15731 (2008).

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 42, pp. 28642–28649, October 16, 2009

Paraquat Toxicity Induced by Voltage-dependent Anion Channel 1 Acts as an NADH-dependent Oxidoreductase*[S]

Received for publication, June 12, 2009, and in revised form, August 7, 2009 Published, JBC Papers in Press, August 28, 2009, DOI 10.1074/jbc.M109.033431

Hiroki Shimada[‡1], Kei-Ichi Hirai[§], Eriko Simamura[‡], Toshihisa Hatta[‡], Hiroki Iwakiri[¶], Keiji Mizuki[¶], Taizo Hatta[¶], Tatsuya Sawasaki[‖], Satoko Matsunaga[‖], Yaeta Endo[‖**], and Shigeomi Shimizu[‡‡]**

*From [‡]Molecular and Cell Structural Science, Kanazawa Medical University, Uchinada, Ishikawa 920-0293, the [§]Niwa Institute for Immunology, Tosashimizu, Kochi 787-0306, the [¶]Department of Nanoscience, Sojo University, Ikeda, Kumamoto 860-0082, the [‖]Cell-free Science and Technology Research Center and the Venture Business Laboratory, Ehime University, Matsuyama, Ehime 790-8577, the **RIKEN Genomic Sciences Center, Tsurumi, Yokohama 230-0045, and the [‡‡]Department of Pathological Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Yushima, Bunkyo, Tokyo 113-8510, Japan*

Paraquat (PQ), a herbicide used worldwide, causes fatal injury to organs upon high dose ingestion. Treatments for PQ poisoning are unreliable, and numerous deaths have been attributed inappropriate usage of the agent. It is generally speculated that a microsomal drug-metabolizing enzyme system is responsible for PQ toxicity. However, recent studies have demonstrated cytotoxicity via mitochondria, and therefore, the cytotoxic mechanism remains controversial. Here, we demonstrated that mitochondrial NADH-dependent PQ reductase containing a voltage-dependent anion channel 1 (VDAC1) is responsible for PQ cytotoxicity. When mitochondria were incubated with NADH and PQ, superoxide anion ($O_2^{\cdot-}$) was produced, and the mitochondria ruptured. Outer membrane extract oxidized NADH in a PQ dose-dependent manner, and oxidation was suppressed by VDAC inhibitors. Zymographic analysis revealed the presence of VDAC1 protein in the oxidoreductase, and the direct binding of PQ to VDAC1 was demonstrated using biotinylated PQ. VDAC1-overexpressing cells showed increased $O_2^{\cdot-}$ production and cytotoxicity, both of which were suppressed in VDAC1 knockdown cells. These results indicated that a VDAC1-containing mitochondrial system is involved in PQ poisoning. These insights into the mechanism of PQ poisoning not only demonstrated novel physiological functions of VDAC protein, but they may facilitate the development of new therapeutic approaches.

Paraquat (PQ^{2+}; methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride) is an effective herbicide used in more than 120 countries (1). Although it is classified as a low hazard compound, PQ is hazardous when used improperly and has been found responsible for thousands of deaths worldwide because of intentional overdose and high levels of occupational and accidental exposure especially in developing countries (1). Direct exposure to PQ causes severe irritation to the eyes and skin, and ingestion of concentrated products may result in fatal injury to lungs because of edema, hemorrhage, and subsequent fibrosis as well as damage to other organs (2). Additionally, PQ has emerged as a risk factor for Parkinson disease (3). The acute toxicity of PQ in mammals is mediated by reactive oxygen species (ROS) produced by a cyclic oxidation-reduction reaction (4). It is generally speculated that NADPH-cytochrome P450 reductase in microsomal drug-metabolizing enzyme systems is responsible for the production of ROS (5). However, we previously observed that the initial ultrastructural alterations associated with PQ exposure occurred only in mitochondria and not in the endoplasmic reticulum in pulmonary cells *in vivo* (6) and *in vitro* (7). In addition, several reports have suggested the cytotoxicity of PQ via mitochondrial dysfunction (8–10). Despite the development of a number of treatments for PQ poisoning, the efficacy and reliability of currently available treatments have remained limited because of an insufficient understanding of PQ cytotoxicity (2).

We recently discovered that active NADH-dependent oxidoreductase located on the mitochondrial outer membrane reduced PQ to a radical form that spontaneously formed superoxide anion ($O_2^{\cdot-}$) and destroyed mitochondria (11–13). Furthermore, we demonstrated that 1) PQ was initially metabolized to monopyridone in the cytosol and subsequently hydroxylated by the microsomes and 2) the induction of drug-metabolizing enzymes and the administration of a ROS scavenger reduced PQ toxicity in mice (11, 14). These results indicate that the mitochondrial system, not the microsomal system, is responsible for PQ toxicity. We verified that enzymes in the electron transport chain and NADH-cytochrome b_5 reductase, an NADH-dependent oxidoreductase in the outer membrane, were not involved in this reaction (11, 12). A voltage-dependent anion channel (VDAC), an abundant pore-forming protein in the outer membrane, exerts numerous physiological functions as a channel; it regulates both the metabolite flux of mitochondria and transmembrane potential, and plays a role in apoptosis. Recently, it was reported that NADH regulates VDAC func-

* This work was supported by Grants-in-aid for Scientific Research 15591664, 17591899, 19390291, and 21791045 from the Japan Society for the Promotion of Science, Grants for Promoted Research S2003-12, S2004-12, C2007-4, S2007-9, C2008-1, S2008-10, C2009-3, and S2009-10 from Kanazawa Medical University, Grant for Project Research H2009-14 from High-Tech Research Center of Kanazawa Medical University, and in part by The Ministry of Education, Culture, Sports, Science, and Technology, Japan Grant S0801085.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental schemes.

[1] To whom correspondence should be addressed. Fax: 81-76-218-8189; E-mail: simada-h@kanazawa-med.ac.jp.

[2] The abbreviations used are: PQ, paraquat; BQ, benzoquinone; DCF, 2′,7′-dichlorofluorescein; DCFH, DCF-diacetate; DIDS, 4,4′-diisothiocyanatostilbene-2,29-disulfic acid; IC_{50}, 50% growth inhibition toxicity; mAb, monoclonal antibody; PTP, permeability transition pore; TBS, Tris-buffered saline; VDAC, voltage-dependent anion channel; ROS, reactive oxygen species; SOD, superoxide dismutase; siRNA, small Interfering RNA.

tion (15), and an isoform of VDAC localized in the plasma membrane possesses NADH-ferricyanide reductase activity (16). Therefore, we attempted to determine whether or not NADH-PQ oxidoreductase on mitochondria is responsible for PQ cytotoxicity and if VDAC participates in this activity.

EXPERIMENTAL PROCEDURES

Cell Line

HeLa cells were provided by RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum at 37 °C in a humidified CO_2 incubator.

Intracellular ROS Production

Mitochondrial superoxide production in HeLa cells was detected using MitoSOX® (Molecular Probes Inc., Eugene, OR), a red fluorescent mitochondrial superoxide indicator, according to the given protocol. Cells were pretreated with 1 mM PQ; Sigma-Aldrich) for 50 min at 37 °C and incubated with 5 μM MitoSOX for 10 min in the dark. The medium was exchanged for fresh medium, and the cells were observed by a fluorescence microscope (Olympus IX70, Olympus Corp., Tokyo, Japan). The effects of benzoquinone (BQ; 0.2 mM, Sigma-Aldrich) were evaluated after 10 min of incubation in BQ-added medium. Intracellular H_2O_2 production in HeLa cells by PQ was detected using 2′,7′-dichlorofluorescein-diacetate (DCFH; Molecular Probes) (17, 18). Briefly, cells were pretreated with 1 mM PQ for 1 h at 37 °C, and then the cells were incubated with 5 μM DCFH for 20 min in the dark. Afterward, the medium was exchanged for fresh medium; fluorescence images that appeared after the formation of 2′,7′-dichlorofluorescein (DCF) were observed by fluorescence microscopy.

Preparation of Mitochondria

Mitochondria were isolated from the livers of male Wistar rats or from HeLa cells by differential centrifugation (11, 12). Mitochondria were suspended in 0.25 M sucrose solution containing 0.05 M Tris-HCl, 20 mM KCl, 2.0 mM $MgCl_2$, and 1.0 mM Na_2HPO_4 (pH 7.4). The mitochondria were starved for 20 min at 37 °C to consume endogenous substrates before use. The Kanazawa Medical University Animal Care and Use Committee approved all studies. All animals were cared for and treated in accordance with the Committee guidelines.

PQ-dependent Hydrogen Peroxide (H_2O_2) Production on Mitochondria

Mitochondria were attached onto a glass-based culture dishes coated with Cell-Tak® (BD Biosciences) (19). The dishes were incubated with 10 mM PQ and 2 mM NADH (Oriental Yeast Co., Ltd., Tokyo, Japan) in the sucrose solution containing 5 μM DCFH, 5 μM rotenone (Sigma-Aldrich), and 1 μM *p*-hydroxymercuribenzoate (Sigma-Aldrich) at 37 °C. Fluorescence images were captured by a digital CCD camera (Pixera Penguin 150 CL, Pixera Corp., Los Gatos, CA) attached to a microscope and were analyzed by Lumina Vision bio-imaging analysis system (Mitani Corp., Fukui, Japan). The fluorescence intensity per 1000 mitochondria was calculated, and the mean value of three areas from each sample was compared. BQ (0.3 mM), anti-VDAC1 monoclonal antibody (mAb; anti-porin 31 HL mAb, 9 μg/ml; Calbiochem), and 4,4′-diisothiocyanatostilbene-2,29-disulfic acid (DIDS; 100 μM, Sigma-Aldrich) were evaluated by addition to the reaction mixture.

Electron Microscopy

Mitochondria were transferred to a sucrose solution containing 3 mM PQ, 2 mM NADH, 5 μM rotenone, 1 μM *p*-hydroxymercuribenzoate, and the solution was reacted for 30 min at 37 °C (11, 12). Superoxide dismutase (SOD; 3000 units/ml, Sigma-Aldrich) effects were evaluated by the addition of SOD to the reaction mixture. Anti-VDAC1 mAb (3 μg/ml) effects were evaluated by preincubation with the mitochondria for 5 min at 37 °C. The reaction was stopped by the addition of cold buffer. Mitochondria were immediately centrifuged, and the packed sediments were covered with 2% glutaraldehyde in phosphate-buffered saline and fixed for 1 h. The fixed clots were prepared for electron microscopy (11) and then observed by a transmission electron microscope (JEM-1200EX, JEOL Co. Ltd, Tokyo, Japan). The percentage of intact mitochondria per area was counted, and the mean of three areas was calculated.

Growth Inhibition Assays

Growth inhibition assays were performed by the stepwise addition of PQ, according to the method described by Saotome (20). Subconfluent HeLa cells were harvested by trypsinization and were precultured on 96-well plates (3×10^3 cells per well) for 24 h. Cells were treated with 7–250 μM PQ and were then cultured for 72 h. The effects of Trolox® (a water-soluble analog of vitamin E; 1 mM, Sigma-Aldrich) were evaluated by its addition to the medium. The 50% growth inhibition toxicity (IC_{50}) was estimated at 72 h.

Extraction of NADH-PQ Oxidoreductase from the Outer Membrane

To extract NADH-PQ oxidoreductase, two-step extraction with Triton X-100, deoxycholate followed by SDS/Igepal® CA-630 was performed (21). The outer membranes were isolated from the mitochondria by discontinuous sucrose gradient centrifugation (12). The isolated outer membranes were suspended in 20 mM Tris-HCl buffer (pH 7.6) containing 1% Triton X-100, 1% sodium deoxycholate, and 1 mM EDTA. The suspensions were left to stand on ice for 1 h. Suspensions were then centrifuged at 105,000 × *g* for 60 min. The precipitates were resuspended in a 20 mM Tris buffer with 0.06% SDS and 0.1% Igepal CA-630. The suspensions were left on ice for 1 h. The supernatants were collected by centrifugation at 105,000 × *g*.

Preparation of NADH-PQ Oxidoreductase Fraction

The supernatants were diluted with 20 mM Tris-HCl buffer (pH 8.0) containing 0.03% Triton X-100 and 10% glycerol, and the dilutions were loaded onto an anion exchange column (DEAE MemSep® 1000; Millipore Corp. Billerica, MA). The columns were washed with the Tris buffer, and proteins were eluted using a NaCl gradient. The fractions containing NADH-PQ oxidoreductase were collected from 0.25–0.3 M