Table 1 Yield, association rate and solubility of synthesized membrane proteins

	Proteins	Anotation	Number of TMD	Molecular weight (kDa)	Yield (μg/150 μl reaction)	Solubility (%)	Association rates with liposomes (%)	Yield × Association rates (μg)
1	Itga1[#]	Integrin	1	131	10.2	38.3	43.3	4.4
2	KCNJ8	Potassium inwardly-rectifying channel	2	48	4.6	22.6	72.6	3.3
3	KCNJ13	Potassium inwardly-rectifying channel	2	41	5.9	23.1	48.7	2.9
4	KCNJ15	Potassium inwardly-rectifying channel	2	43	12.5	10.5	58.8	7.3
5	P2RX1	Purinergic receptor	2	45	4.7	46.0	65.4	3.1
6	P2rx2[#]	Purinergic receptor	2	55	5.0	40.4	58.3	2.9
7	P2RX4	Purinergic receptor	2	43	6.1	30.5	65.4	4.0
8	P2RX5	Purinergic receptor	2	47	5.5	50.0	58.2	3.2
9	GRIA2	Glutamate receptor	3	99	7.3	24.1	59.8	4.4
10	CACNG3	Voltage-dependent calcium channel	4	36	0.9	54.1	62.7	0.6
11	CACNG4	Voltage-dependent calcium channel	4	37	0.4	61.0	70.8	0.3
12	GABRA3	GABA receptor	4	55	11.0	17.1	58.1	6.4
13	GABRB1	GABA receptor	4	54	4.9	21.0	60.9	3.0
14	GABRD	GABA receptor	4	51	8.4	12.5	58.8	4.9
15	GABRG1	GABA receptor	4	54	7.0	6.9	48.6	3.4
16	Glra1[#]	Glycine receptor	4	52	5.4	17.1	67.6	3.6
17	AQP3	Aquaporin	6	32	0.4	21.4	66.6	0.3
18	EDNRA	Endothelin receptor	7	49	11.1	8.3	56.6	6.3
19	EDNRB	Endothelin receptor	7	50	3.9	28.3	68.3	2.7
20	GPR37	G protein-coupled receptor 37	7	67	0.5	33.0	58.8	0.3
21	HTR2B	5-Hydroxytryptamine receptor	7	54	2.2	57.0	55.8	1.2
22	P2RY2	Purinergic receptor	7	42	7.4	18.9	62.4	4.6
23	P2RY10	Purinergic receptor	7	39	7.5	14.0	61.2	4.6
24	P2RY11	Purinergic receptor	7	40	3.8	26.3	51.7	2.0
25	P2RY13	Purinergic receptor	7	38	8.3	12.7	64.8	5.4
26	P2RY14	Purinergic receptor	7	39	5.6	14.3	53.5	3.0
27	SLC1A7	Glutamate transporter	10	61	6.7	42.9	69.4	4.6
28	SLC6A3	Dopamine transporter	12	69	0.7	9.1	44.4	0.3
29	SLC6A13	GABA transporter, GABA	12	68	5.5	38.1	51.1	2.8
30	SLC6A18	Solute carrier family 6, member 18	12	71	13.1	15.3	48.1	6.3
31	Slc18a2[#]	Vesicular monoamine transporter	12	56	10.9	27.1	56.3	6.2
32	SLC22A4	Organic cation transporter	12	62	4.4	33.1	60.2	2.6
33	SLC22A7	Organic anion transporter	12	60	0.8	56.2	62.7	0.5
34	SLC22A8	Organic anion transporter	12	60	5.6	36.0	56.9	3.2
35	SLC22A11	organic anion/cation transporter	12	60	7.1	14.1	62.7	4.5
36	SLC22A12	Organic anion/cation transporter	12	60	6.2	16.4	62.3	3.9
37	Slc5a1[#]	Sodium/glucose cotransporter	14	73	10.6	21.8	53.4	5.7
38	SLC5A6	Sodium-dependent vitamin transporter	14	69	5.1	28.7	62.1	3.2
39	SLC5A10	Sodium/glucose cotransporter	14	62	4.1	42.2	47.5	1.9
40	Slc7a1[#]	Cationic amino acid transporter	14	67	6.7	23.3	57.3	3.8

[#] Mouse clone.

The extent of association of all 40 proteins tested, containing between one and 14 TMDs, were from 43 to 73% and the mean value was 59% (Table 1). The amount of proteins associated with liposomes was calculated from the yield and extent of association for each protein (Figure 3C and Table 1). The mean value of the association yield was 3.4 μg/150 μL reaction. When analyzing association by the number of TMDs, the lowest extent of association was 43% for 1 TMD (Itgal), whereas the mean value of association for proteins having more than 2 TMDs was approximately 60% (Table 1). These results indicate that efficiency of association of proteins having only 1TMD might be lower than that of proteins having more than 2TMD.

Figure 3D shows the relationship between solubility and association rate of tested proteins. Solubility of MPs synthesized using the wheat cell-free system in the absence of liposomes ranged from eight to 61%. The extent of association of these proteins ranged from 40 to 70% regardless of their solubilities. On the other hand, soluble proteins showed more than 80% solubility and their association rates were less than 20%, indicating that the extent of liposome association for soluble proteins is much lower than that of MPs. In comparison to the tested soluble proteins, the MPs examined had a wide variety of sizes, functional roles and topologies, and all of them appeared to be efficiently associated with liposomes during cell-free synthesis. Moreover, there were no significant relationship between the extent of protein association and their respective yields (Figure 3E).

In this experiment, we found that a variety of MPs make complexes with lipids during wheat cell-free synthesis in the presence of liposomes. It is not yet clear whether these MPs are integrated into liposomes or attached to surface of liposomes with their hydrophobic regions. For replying to this question, further experiments for evaluating function and/or structure of each synthesized MP are needed. However, as association of MPs with liposomes prevents aggregation and precipitation of synthesized MPs during synthesis reaction, formation of lipid/MP complex would be an important step for preparing MPs.

Katzen et al. [20] recently reported that insertion of EmrE into a discoidal membrane scaffold in correctly folded state during cell-free synthesis by analysis of binding activity of its substrate, tetraphenylphosphonium. Moritani et al. [21] demonstrated that connexin-43 synthesized by an *E. coli* cell-free system, PURE system containing minimum protein synthesis factors [22], in the presence of liposomes is directly integrated with a uniform orientation into liposome membrane. The connexin-43 synthesized into liposomes was shown to deliver a hydrophilic and bioactive oligo-peptide to cells through gap junctions [23]. They speculate that liposomes may have chaperone-like function because their system consists of only protein synthesis factors and liposomes [21]. Although it is still not clear any kinds of MPs can receive such a chaperone-like effect from liposomes, the addition of artificial membranes to cell-free reactions appears to be effective in synthesizing membrane-stabilized MPs.

Partial purification of synthesized MPs by density-gradient ultracentrifugation

In this study, we demonstrated that a variety of mammalian MPs are efficiently associated with liposomes during wheat cell-free synthesis supplemented with liposomes. Next, we tried to partially purify the synthesized lipid/MP complexes from the endogenous wheat germ proteins. For this experiment we used P2RX4 as a model protein, because both the yield (6.1 μg) and extent of association (66%) are very close to the mean value for both of these parameters in the full mammalian MP dataset above (5.9 μg and 59%, respectively).

A GFP-P2RX4 fusion protein was synthesized using the wheat cell-free system supplemented with liposomes and the resultant lipid/MP complexes were subjected to Accudenz DGU. Accudenz is a non-toxic medium used for fractionation of proteins, organelles, and cells [15,24-26]. The wheat cell-free synthesis reaction was brought to 40% Accudenz by addition of an 80% Accudenz solution, placed at the bottom of an ultra-centrifugation tube, and overlaid with 35% Accudenz solution, 30% Accudenz solution and DGU solution. It is expected that after Accudenz DGU, liposomes float to the top of the centrifuge tube owing to their low density [15,24]. As shown in Figure 4A, the fluorescence from GFP-P2RX4 fusion proteins was observed at the top of the gradient after Accudenz DGU, while the majority of the fluorescence from a soluble GFP control remained at the bottom. After fractionation, the fluorescence of each fraction was measured with a spectrofluorometer (excitation 480 nm/emission 510 nm). High fluorescence intensity at the top fraction, associated with floated liposomes, was detected in GFP-P2RX4 sample synthesized by liposome supplemented wheat cell-free synthesis (Figure 4B). In the other samples, GFP synthesized with or without liposomes and GFP-P2RX4 synthesized without liposomes, fluorescence was mainly detected in the lower fractions (Figure 4B). The extent of association of GFP-P2RX4 in this experiment (66%: Fraction 1 and 2) was similar to the association of P2RX4 calculated by sucrose DGU (66%). Although we also tested association of P2RX2-GFP with liposomes with same procedure, there were no significant differences in association rate (data not shown).

Although fluorescence was seen at the top of the gradient in the soluble GFP control after Accudenz DGU

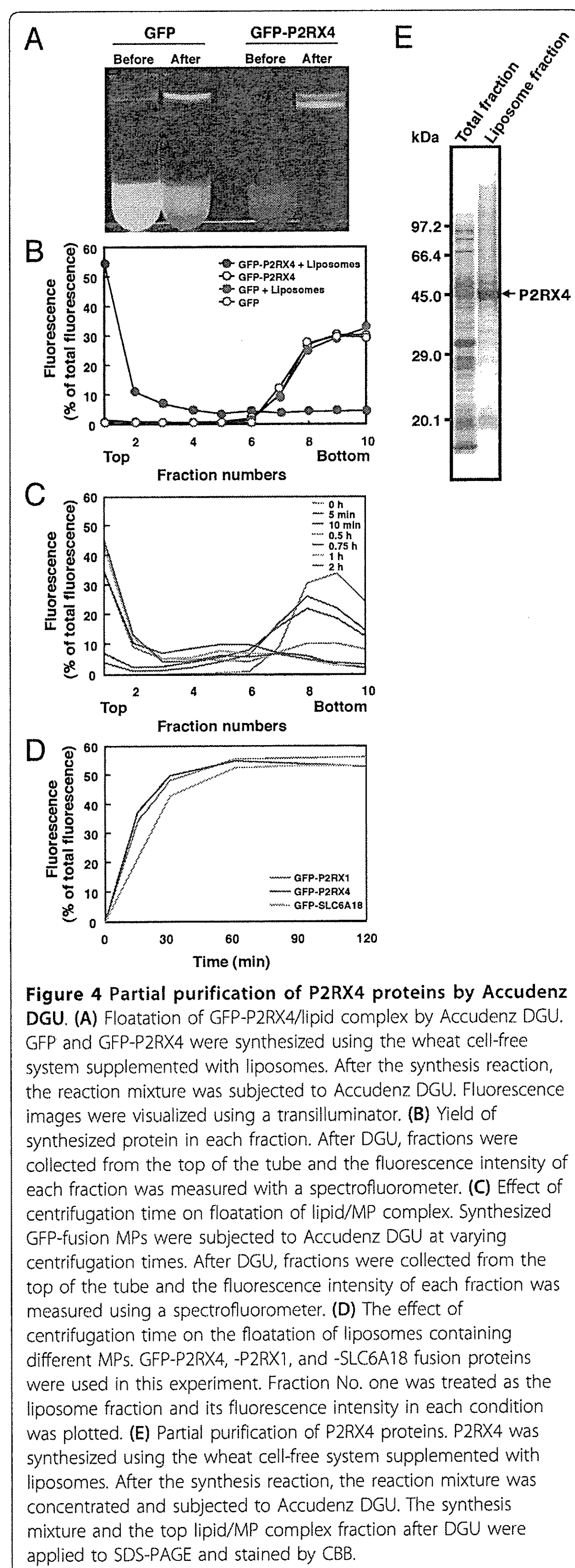

Figure 4 Partial purification of P2RX4 proteins by Accudenz DGU. **(A)** Floatation of GFP-P2RX4/lipid complex by Accudenz DGU. GFP and GFP-P2RX4 were synthesized using the wheat cell-free system supplemented with liposomes. After the synthesis reaction, the reaction mixture was subjected to Accudenz DGU. Fluorescence images were visualized using a transilluminator. **(B)** Yield of synthesized protein in each fraction. After DGU, fractions were collected from the top of the tube and the fluorescence intensity of each fraction was measured with a spectrofluorometer. **(C)** Effect of centrifugation time on floatation of lipid/MP complex. Synthesized GFP-fusion MPs were subjected to Accudenz DGU at varying centrifugation times. After DGU, fractions were collected from the top of the tube and the fluorescence intensity of each fraction was measured using a spectrofluorometer. **(D)** The effect of centrifugation time on the floatation of liposomes containing different MPs. GFP-P2RX4, -P2RX1, and -SLC6A18 fusion proteins were used in this experiment. Fraction No. one was treated as the liposome fraction and its fluorescence intensity in each condition was plotted. **(E)** Partial purification of P2RX4 proteins. P2RX4 was synthesized using the wheat cell-free system supplemented with liposomes. After the synthesis reaction, the reaction mixture was concentrated and subjected to Accudenz DGU. The synthesis mixture and the top lipid/MP complex fraction after DGU were applied to SDS-PAGE and stained by CBB.

by using a transilluminator (excitation: 400-500 nm) with a filter for removing blue light (Figure 4A), the fluorescence in the top fraction was not detected with a spectrofluorometer using a condition for detection of fluorescence from GFP (excitation 480 nm/emission 510 nm) (Figure 4B). These results indicate that the fluorescence in the top fraction is not attributed to GFP but probably to the liposomes. In comparison to sucrose DGU, Accudenz DGU makes lipid/MP complex recovery easier due to the fact that liposomes float in Accudenz as opposed to sinking as in sucrose. Additionally, Accudenz is a preferred DGU solution because it is non-toxic.

The GFP-fusion P2RX4 is an ideal construct to determine the optimal conditions for Accudenz DGU as the liposome-associated protein can be readily monitored by fluorescence. We tested several centrifugation times for Accudenz DGU. As shown in Figure 4C, the fluorescence in the top fraction gradually increased in proportion centrifugation time. One-h centrifugation appears to be sufficient for lipid/MP complex floatation. The results from Accudenz DGU of two additional MPs, GFP-P2RX1 and GFP-SLC6A18, also showed that the floatation rates of lipid/MP complex plateaus at a one-h centrifugation (Figure 4D).

As shown in Figure 4B, GFP and GFP-P2RX4 could be clearly separated by DGU. The result suggests that lipid/MP complexes can be separated from endogenous proteins derived from the wheat germ extract by Accudenz DGU. Nomura et al. also reported that cytochrome b5 synthesized by the wheat cell-free system in the presence of liposomes was easily purified by simplified discontinuous DGU [27]. In addition, Goren and Fox showed that a MP, hSCD1, synthesized by the wheat cell-free system supplemented with liposomes could be separated from endogenous wheat germ proteins by Accudenz DGU [15]. We next tried to purify P2RX4, without a GFP fusion, by this method. After synthesis supplemented with liposomes, lipid/MP complexes were concentrated by centrifugation and applied to Accudenz DGU. After ultracentrifugation, the top fraction was recovered and applied to SDS-PAGE. As shown in Figure 4E, P2RX4 was detected as a major band by CBB-staining after SDS-PAGE. This result indicates that lipid/MP complexes synthesized by our wheat cell-free system supplemented with liposomes can be partially purified by Accudenz DGU. To test whether proteins purified by this method are functional, we tested the activity of AtPPT1 partially purified as lipid/MP complexes. We detected transport activity of AtPPT1 purified with Accudenz DGU (data not shown). In addition to functional analysis, this partially purified protein could be used for structural analysis. However, some contaminating proteins from wheat germ extracts do

float with the lipid/MP complexes. Goren and Fox [15] reported that, along with other unclassified contaminants, HSP70, elongation factor 1 α and 16.9 kDa heat shock protein were seen in wheat cell-free synthesized and floated lipid/MP complexes. For functional and structural analysis, further purification steps would be required including affinity-tag purification and gel-filtration.

Conclusion

We have developed a production method for lipid/MP complexes using a wheat germ cell-free system supplemented with liposomes. Using this method, a variety of mammalian MPs were efficiently associated with liposomes co-translationally. The resultant lipid/MP complexes are easily separated from other proteins in wheat germ extract by DGU. This synthesis method is useful in the preparation of MP for structural and functional analysis.

Methods

Wheat Cell-free protein synthesis

Details of the wheat cell-free reaction were described in previously [28-30]. The 40 MPs and five soluble proteins in this study were selected from the Mammalian Gene Collection and FANTOM collection (Danaform, Yokohama, Japan). The unique primers for each protein (Additional file 1 Table S1) were designed and templates for transcription were made by the split-primer PCR method as described previously [18,19]. The first PCR was performed with 10 nM of the gene specific primer (Additional file 1 Table S1) and 10 nM of the AODA2303 primer (5'-GTCAGACCCCGTAGAAAAGA) or 10 nM AODS primer (5'-TTTCTACGGGGTCTGACGCT). The second PCR was amplified with 100 nM SPu primer (5'-GC GTAGCATTTAGGTGACACT), 1 nM deSP6E01-S1 primer (5'-GGTGACACTATAGAACTCACCTATCTCC CCAACACCTAATAACATTCAAT CACTCTTTCCAC-TAACCACCTATCTACATCACCAACCACCCACCAC-CACCAATG), and 100 nM AODA2303 primer or 100 nM AODS primer. mRNA was prepared by *in vitro* transcription in a reaction volume of 100 μl and was purified by ethanol precipitation. The mRNA pellet was resuspended in 30 μl of water. The translation reaction was performed using the bilayer method, supplemented with [^{14}C-leucine (50 μCi/ml, GE Healthcare, Tokyo, Japan) [31], in which a 25 μl translation layer was overlaid with a 125 μl substrate feeding buffer. Asolectin liposomes were prepared as described previously [16,32] and added to both layers (10 mg/ml final concentration). The bilayer reaction was incubated at 26°C for 16 h. After the reaction, the amount of [^{14}C]-leucine incorporation into synthesized proteins, an indicator of synthesis yield, was determined by 10% trichloroacetic acid precipitation and liquid scintillation spectroscopy.

Sucrose density gradient ultracentrifugation

Lipid/MP complexes were separated from proteins in the wheat germ extract by sucrose DGU. Synthesized proteins (100 μl) were loaded onto a discontinuous sucrose gradient consisting of 1,300 μl of 10% (w/v) and 600 μl of 30% (w/v) sucrose in DGU solution containing 140 mM NaCl, 5.4 mM KCl and 10 mM Tris-HCl (pH 8.0). The gradient was centrifuged at 105,000 *g* for 4 h at 4°C in a Hitachi S55S rotor (Hitachi High-Technology, Tokyo, Japan). Fractions were collected from the top of the tubes, and the protein content of each fraction was estimated by measurement of the amount of radioactivity associated with proteins precipitated by 10% trichloroacetic acid.

Transport assay of phosphate translocator

Liposomes were prepared from acetone-washed asolectin (Sigma-Aldrich, Tokyo, Japan) by sonication for 5 min at 4°C in water. AtPPT1 was synthesized by wheat cell-free system with (10 mg/ml final concentration) or without liposomes. Half of the AtPPT1 synthesized without liposomes was mixed with liposomes (10 mg/ml final concentration) after the synthesis reaction and incubated for 30 min at 26°C. These reaction mixtures were desalted by gel filtration with a Micro-Spin G-25 column (GE Healthcare) that had been equilibrated with 10 mM Tricine-KOH (pH 7.6).

The amount of protein synthesized in the cell-free system was estimated from the incorporation of [^{14}C]-leucine. After the reaction, the amount of [^{14}C]-leucine incorporation into synthesized proteins, an indicator of synthesis yield, was determined by 10% trichloroacetic acid precipitation and liquid scintillation spectroscopy.

Substrate-including liposomes (80 mg/ml final concentration) were prepared from acetone-washed asolectin by sonication for 5 min at 4°C in a solution containing 200 mM Tricine-KOH (pH 7.6), 40 mM potassium gluconate and 60 mM potassium phosphate (substrate). Desalted reaction mixtures were mixed with the substrate-preloaded liposomes, frozen in liquid nitrogen, thawed at room temperature and sonicated for 18 sec. Substrate that remained outside of the liposomes was removed with a Dowex AG-1X8 column (Bio-Rad, Tokyo, Japan) that had been equilibrated with a solution containing 100 mM sodium gluconate, 40 mM potassium gluconate and 10 mM Tricine-KOH (pH 7.6). The liposome mixture was applied to the column and eluted with the equilibration solution.

Transport reactions were initiated by the addition of 13 μl of [^{32}P] Pi (GE healthcare) to 300 μl of liposomes

(final phosphate concentration, inside: 30 mM, outside: 0.5 mM). The assay was performed at 25°C for 2 min, and the reaction was terminated by application of the reaction mixture to a Dowex AG-1X8 column that had been equilibrated with 150 mM sodium acetate. The radio-activity associated with the eluted liposomes was measured with a liquid scintillation spectrometer.

Plasmid construction

GFP fragments were amplified by PCR with the primers EcoRV-GFP-5' (5'-GAGAGATATCATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAG TGGCACGAAGGTGGAGGTGGAATGGTGAGCAAGGGCGAGGA-3') and GFP-NotI-3' (5'-TCTCGCGGCCGCTCCACCTCCACCCTTGTACAGCTCGTCCATGC-3). The PCR fragments were digested with *Eco*RV and *Not*I and then cloned into the corresponding sites in the pEU-E01-MCS vector (Cellfree Sciences, Matsuyama, Japan). The resultant plasmid was designated pEU-E01-GFP-N. P2RX4, P2RX1, and SLC6A18 were obtained from cDNA clones and amplified by PCR with the primer sets NotI-P2RX4-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCGGGCTGCTGCGCCGC) and P2RX4-SalI-3' (5'-AGAGGTCGACTCACTGGTCCAGCTCACTAG), NotI-P2RX1-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCGGGCTGCTGC GCCGC) and P2RX1-SalI-3' (5'-AGAGGTCGACTCACTGGTCCAGCTCACTAG), and NotI-SLC6A18-5' (5'-GAGAGCGGCCGCTGAAAACCTGTATTTTCAGGGCATGGCTCATGCCCCAGAACC) and SLC6A18-SalI-3' (5'-AGAGGTCGACTCAGCGCATGTCCGTGTCCG), respectively. These PCR fragments were digested with *Not*I and *Sal*I and then cloned into the *Not*I-*Sal*I sites of pEU-E01-GFP-N. The resultant plasmids were designated pUE-E01-GFP-P2RX4, pUE-E01-GFP-P2RX1, and pUE-E01-GFP-SLC6A18, respectively, and used for production of GFP-fusion proteins. The P2RX4 ORF was amplified by PCR using the primer pair P2RX4-EcoRV-5' (5'-GAGAGATATCATGGCGGGCTGCTGCGCCGC) and P2RX4-NotI-3' (5'-CTCTGCGGCCGCTCCACCTCCACCCTGG CCAGCTCACTAGCAA). The PCR product was digested with *Eco*RV and *Not*I and then inserted into *Eco*RV-*Not*I sites of pEU-E01-MCS. The resultant plasmid was designated pEU-E01-P2RX4. The nucleotide sequences of each DNA fragment amplified by PCR was confirmed by DNA sequencing.

Detection and quantification of fluorescence from GFP proteins

Fluorescence images were visualized with a transilluminator (excitation: 400-500 nm, Dark Reader DR45M, Clare Chemical Research, Dolores, CO). Fluorescence intensity from GFP proteins was measured by a, Wallac 1420 Multilabel Counter spectrofluorometer (Perkin-Elmer Japan, Chiba, Japan).

Partial purification of P2RX4

mRNA was prepared by *in vitro* transcription using pEU-E01-P2RX4 as the template. The translation reaction was performed in the presence of liposomes (10 mg/ml final concentration) using the bilayer method with a bottom layer of 500 μl and an upper layer of 5500 μl. After the synthesis reaction, the reaction mixture was concentrated to 300 μl with a concentrator (Amicon Ultra-15, 30,000 MWCO, Millipore-Japan, Tokyo, Japan). For Accudenz (Accurate Chemical and Scientific, Westbury, NY) DGU, Accudenz was dissolved into DGU solution to make 30, 35, and 80% (w/v) Accudenz solutions. Three hundred μl of concentrated sample was mixed with 300 μl of 80% (w/v) Accudenz solution. The resultant 40% Accudenz solution containing the synthesized protein was placed in the bottom of a centrifuge tube, and overlaid with 650 μl of 35% (w/v) Accudenz solution, 650 μl of 30% (w/v) Accudenz solution, and 100 μl of DGU solution. The gradient was centrifuged at 105,000 *g* for 4 h at 4°C in a Hitachi S55S rotor (Hitachi High-Technology, Tokyo, Japan).

Additional material

Additional file 1: Table S1.

Abbreviations

CBB: Coomassie Brilliant Blue; DGU: density gradient ultracentrifugation; hSCD1: human stearoyl-CoA desaturase 1; Liposomes: exogenous liposomes; MP: membrane protein; PCR: polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMD: transmembrane domain

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Authors' contributions

YE and TS designed the experiment. AN, TO, SM, and TI performed the experiments.AN and TS wrote the manuscript. All authors read and approved the final manuscript.

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Wheat germ cell-free protein production system for post-genomic research

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Genomic information becomes useful knowledge only when the structures and functions of gene products are understood. In spite of a vast array of analytical tools developed for biological studies in recent years, producing proteins at will is still a bottleneck in post-genomic studies. The cell-free protein production system we developed using wheat embryos has enabled us to produce high quality proteins for genome-wide functional and structural analyses and at the same time circumvent almost all the limitations, such as biohazards and costs, that have hampered conventional cell-free protein synthesis systems. In the present article, we introduce examples of our new wheat germ cell-free protein production system and its application to functional and structural analyses, with the focus on the former.

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Introduction

The use of genomic information for life science studies and industrial applications requires that the information be translated to produce proteins for functional and structural analyses. Producing proteins at will, however, remains a serious bottleneck. Conventional protein production methods using living cells are, by nature, unable to produce proteins that disturb physiological functions of host cells. In fact, there are many proteins that the conventional methods cannot handle. Furthermore, cell culture and gene expression in conventional *in vivo* systems require trial-and-error by skillful hands and are not therefore suitable for comprehensive parallel protein production. By contrast, organic

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synthesis is impractical because of the extremely low yields and low qualities of synthesized proteins. The advent of cell-free methods that combined biological organisms' inherent capabilities with chemical technology raised expectations overcoming such limitations. They actually proved to be a powerful tool for tracer experiments at the dawn of life sciences, but the low yield resulting from their unstable translation reactions made them inadequate for protein production [1,2]. Our study on the cause of translation instability that had hampered the conventional cell-free systems resulted in the development of a new wheat germ cell-free system. We found that a set of translation machinery extracted from purified wheat embryos permitted stable translation, and we succeeded in creating a new cell-free protein synthesis method which was as efficient as living cells [3]. We then developed various elemental techniques to transform the new cell-free method into a practical *in vitro* protein production system [4]. The resultant wheat germ cell-free system met regulatory requirements related to biohazards and bioethics. Subsequent development of the system included full automation of protein synthesis, which has facilitated the adoption of the system worldwide.

In this article, we review the new wheat cell-free protein production technology and introduce examples of its application to functional and structural protein analyses, including part of our recent progress in comprehensive functional analysis of gene products. The details of the development of this technology and the protein production protocols for its use have been presented in previous reports [5,6].

Development of a practical wheat germ cell-free protein production system

Preparation of highly efficient cell-free translation solution made from wheat embryos

We succeeded in resolving the common problem of conventional cell-free protein synthesis systems, namely, instability. The key to the success was the discovery of the cause of such instability, which followed our findings [7] with respect to the molecular mechanisms of protein synthesis inhibition by plant poisons including ricin, an infamous poison used for assassination. Those findings are summarized as follows:

(1) Although the translation machinery is a robust system by nature, living organisms contain translation auto-inactivation factors that target their own translation apparatus, some of which might work as self-defense agents against pathogens. We found that such factors consisted of ribosome inactivating proteins [8], translation initiation factor modifying enzyme, deoxyribonucleases, ribonucleases, and proteases.

(2) When cells are damaged by homogenization, those translation inactivation factors contaminated from endosperm of wheat seeds during isolation of embryos are triggered to inactivate the translation machinery. This may be attributable to a glitch of the defensive response. Following these findings, we searched for a raw material whose translation machinery was highly stable and rich in content and whose translation inactivation factors were easy to remove. As a result, we found that wheat embryos fully satisfied those requirements, and subsequently succeeded in producing a stable translation solution from the embryos [3]. This work also confirmed that the activation of the translation auto-inactivation mechanism, which may be called a suicide mechanism, was the main cause of the low protein yields that may be a general effect in conventional cell-free protein synthesis systems.

Development of elemental techniques and completion of the wheat germ cell-free system

The translation machinery obtained by removing the translation-inhibiting contaminants from wheat embryos enabled us to optimize the translation constituents and the reaction conditions. We then developed elemental techniques, which in combination completed a practical cell-free protein production system. They included the Split-PCR method for constructing transcription templates, the transcription reaction method, 5′- and 3′-untranslated regions of mRNA that enhance translation, the pEU vector dedicated to the wheat germ cell-free system, and the translation reaction method [4]. Among the pEU vectors we have developed so far, BISHOP-vector is especially useful for genome-wide preparation of tag-free, highly purified proteins [9].

Advantages and characteristics of the wheat germ cell-free protein production system

Overcoming the barrier against conventional cell-free protein synthesis systems, the wheat germ cell-free protein production system has proved to be effective both in comprehensive parallel protein synthesis and in large-scale protein production at a gram level. In manual synthesis using a dialysis bag as a reaction vessel adapted to a principle of continuous flow cell-free translation method [10], the reaction solution containing the translation machinery from purified embryo was able to produce as much as 10 mg of a protein per 1 mL of the reaction volume [4]. One of the most important qualification criteria for a protein production system is that it can produce active proteins in their correct conformation. In this regard, the wheat germ cell-free system outperforms other well-known cell-free systems, such as those based on *Escherichia coli* cells and rabbit reticulocytes (Table 1) with the following advantages:

(a) *Capability to produce high quality eukaryotic multi-domain proteins:* In eukaryotic translation systems, the measured polypeptide growth rate is 3–5 peptide bonds per second. At this rate, folding of the growing polypeptide is co-translational and takes place as it is ejected from the hydrophobic ribosome tunnel into hydrophilic environment. In prokaryotic translation systems, by contrast, polypeptides grow almost ten times faster. Because of this, folding to produce higher-order structures is thought to be post-translational and, with the help of chaperones, take place after elongation of the polypeptide chain is completed and the chain leaves the ribosome [11]. In an *E. coli*-based translation system, whether it be a cell-based recombinant system or a cell-free system, synthesized eukaryotic multi-domain proteins often come out as insoluble aggregates. This is because, in this prokaryotic system, elongation of the polypeptide chains of those proteins occurs too fast for their correct folding.

TABLE 1

Characteristics of the new wheat germ cell-free translation system

	Wheat embryos	*E. coli* cells	Rabbit reticulocytes
Productivity (mg per ml)	10	6	μg order
Folding	Co-translation	Post-translation	Co-translation
Quality	High	Low	High
Codon preference	Loose	Tight	Tight
Reaction temperature (°C)	Wide (4–30)	37	30
Post-translational modification	Yes	No	Yes
Protein complex	Yes	No	No
Membrane protein	Yes	Yes	Yes
Disulfide bond formation	Yes	Yes	No
Biohazard	No	Yes	Yes
Ethical issues	No	Yes	Yes
Cost	Low	Low	High

(b) *mRNA's low codon preference:* This permits high-throughput protein production of falciparum malaria of high A/T composition and *Thermus thermophilus* of high G/C composition without any adjustment of codon usage [12,13]. Introduction of disulfide bonds is also possible [14].

(c) *Safety:* Being made from grain, the wheat germ cell-free system is free from biohazard and bioethical issues. Throughout the entire process from the making of the translation solution to synthesized proteins, bio-pollution is controlled to the minimum.

Furthermore, wheat germ extract contains N-terminal methionine excision activity for protein maturation [15] and N-myristoylation activity [16], additional important characteristics of the system to ensure correct folding. By contrast, N-glycosylation requires addition of endoplasmic reticulum of canine pancreas.

Protein production protocols

Integrating the elemental techniques described above, we completed two protocols for protein production with the wheat germ cell-free system, protocol (A) for comprehensive parallel production of a large variety of samples in small quantities for functional analysis, and protocol (B) for large-scale production for structural analysis (Fig. 1). Protocol (A) describes (a) selection of target genes, (b) construction of transcription templates by the Split-PCR method and subsequent mRNA synthesis, and (c) protein synthesis for functional or other kinds of screening. In most cases, 20-μL reaction volumes using a 96-well titer plate produce enough products for measurements of enzymatic activity. Protocol (B) describes (a) transcription using the pEU vectors containing target genes, and (b) translation by the bilayer method or an intermittent reaction method in which the reaction solution is exchanged at prescribed intervals. The stable translation capability of wheat embryo made it possible to automate protein production; a series of robots have been developed and are commercially available (http://www.cfsciences.com/). Among them, GenDecoder can produce 384 proteins in an overnight campaign, Protemist DTII can synthesize and purify 6 protein samples (0.5 mg each) overnight, and Protemist XE can produce 1 g of a protein in a 36-hour campaign.

Application of the system for high-throughput functional protein analysis

Protein factory

To use the reservoir of genomic information, which had been accumulated over years, we constructed for protein studies a platform for high-throughput protein production and functional analysis, which in turn was used to establish a human protein factory. The first step toward the human protein factory was to construct 33,275 Gateway entry clones from full-length human cDNA libraries and Gateway destination vectors containing various fusion tags. [Gateway entry clones are available to all qualified potential users from the National Institute for Technology and Evaluation (Japan) Biological Resource Center (http://www.nbrc.nite.go.jp/e/hgentry-e.html)]. Of those, 13,364 clones were subjected to protein synthesis in the wheat germ cell-free system using mRNA produced with transcription templates prepared by PCR. All the 75 phosphatases selected from the template collection were successfully produced and purified using a GST fusion tag. Of the 75 phosphatases, 58 showed activity to p-nitrophenyl phosphate, an artificial substrate, and two of them in particular showed high substrate specificity to a known phosphoprotein. Cytokines were synthesized in dithiothreitol-free translation solution to promote the formation of disulfide bonds. The synthesized cytokines showed as much biological activity as commercially available counterparts. Furthermore, the wheat germ cell-free protein production technology has been successfully used in combination with 13,277 Gateway destination vectors to develop protein active microarrays to detect antigen–antibody reactions and measure enzyme activity [14]. Protein mass spectrometry has become a powerful tool in proteome research, but there remain some serious limitations, quantification of proteins being one of them. This was recently solved by the absolute quantification method named FLEXIQuant (Full-Length Expressed Stable Isotope-labeled Proteins for Quantification) developed by Singh *et al.* [17]. The internal standards that form

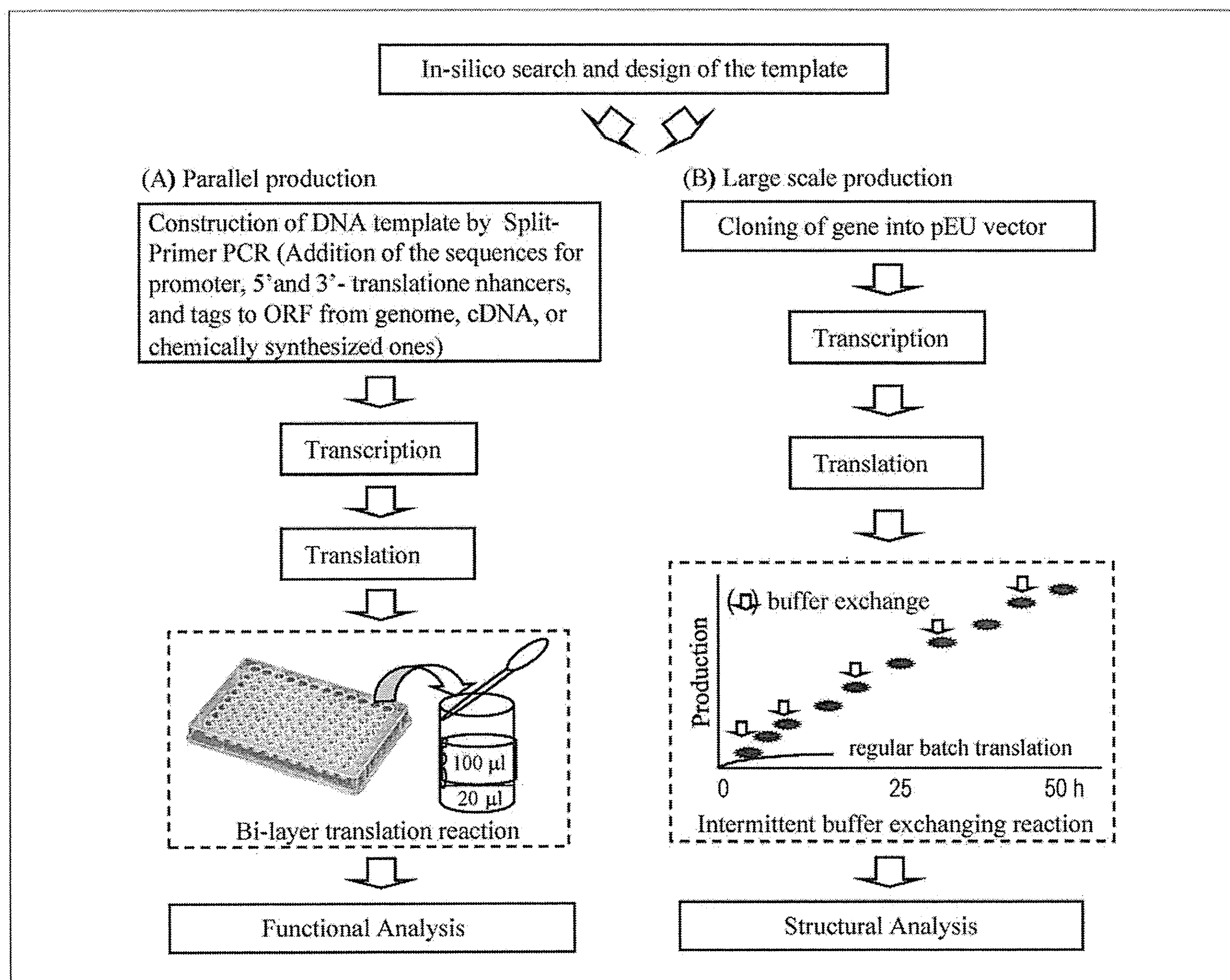

FIGURE 1

Protocols for protein production based on the wheat germ cell-free system. **(a)** Genes of interest are selected from DNA databases and transcription templates are constructed using the Split-PCR method. A typical DNA used for amplification comprises an SP6 promoter, an open reading frame, a reporter, and/or a purification tag (SP6-Tag1-ORF-Tag2). After transcription, the solution is directly used as the mRNA source in the bilayer translation system. **(b)** Suitable gene products selected in (a) can be produced in large quantities. Genes are cloned into a preferred type of pEU plasmid and the mRNA is transcribed. Before translation, the conditions (e.g. ion concentrations and incubation temperature) should be optimized in a small scale, and proteins are produced by intermittent buffer exchange reaction.

the core of FLEXIQuant are ^{15}N- and ^{13}C-labeled full-length proteins of high quality produced in the wheat germ cell-free system.

Method for comprehensive functional analysis

Although a variety of methods using sophisticated instruments to measure protein–protein interaction have been commercialized in recent years, their utility in comprehensive functional analysis is still limited. One issue associated with them is that protein samples are immobilized on beads or other substrates and another is the way samples are prepared and stored. The first issue is exemplified by the protein active microarray described above. It uses beads to immobilize proteins and, as a result, interaction reactions between these immobilized proteins and free proteins take place through the solid phase. The second issue is exemplified by the conventional biochemical method (radioactive isotope labeling) we have used for identifying protein kinases [18] or searching for substrate proteins [19] using the wheat germ cell-free system. This method is not suitable for high-throughput, high sensitivity detection. To circumvent those issues, we developed a method that combines the wheat germ cell-free protein synthesis technology with the AlphaScreen™ (PerkinElmer) detection technique. An example of its application is our ongoing comprehensive search for protein–protein interaction as illustrated in Fig. 2 and is described below.

Step 1: Generate DNA templates in either of the two types, one encoding a biotin-ligating peptide at the N-terminus and the other having FLAG-tag at the C-terminus, which are to bind to donor and accepter beads, respectively. After transcription, dispense each of the mRNA-containing solutions into one of the wells of a 96-well titer plate filled with the translation mixture and carry out

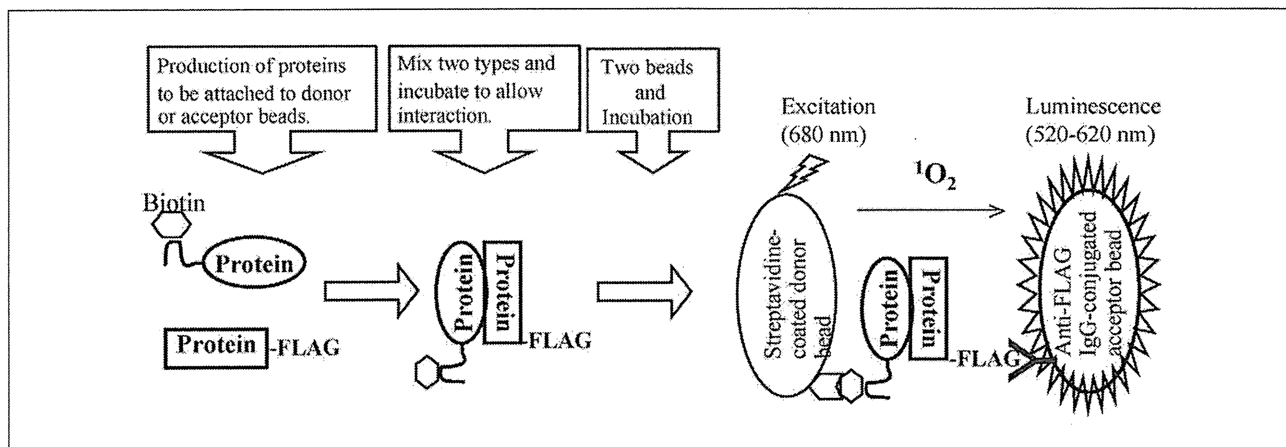

FIGURE 2
Schematic diagram for the functional analysis method based on the wheat germ cell-free protein production system and AlphaScreen^{TM}.

protein synthesis. For the production of biotin-labeled protein, carry out translation in the presence of biotin and biotin ligase produced beforehand in the wheat germ cell-free system.

Step 2: Dilute the translation solutions, add samples containing unpurified proteins of interest prepared in the type opposite to the one selected in Step 1, then incubate to allow interaction or reaction between two protein molecules in each well.

Step 3: Add commercially available AlphaScreen^{TM} reagents (Streptavidin-coated donor beads and Protein A-conjugated acceptor beads) and measure the intensity of the fluorescence. When donor beads and acceptor beads come close within 100–200 nm, singlet oxygen is ejected from donor beads by laser, excites the acceptor beads, and releases fluorescence (Refer to the company's catalog).

Although its operating principle allows only semi-quantitative analysis, this method makes it possible to use high quality, unpurified protein samples produced by the wheat germ cell-free system and permits comprehensive detection of interactions between molecules in the liquid phase. It is also adaptable to other analytical methods such as analysis of ubiquitin complex [20] and single molecule fluorescence spectroscopy (Olympus, Japan) [21], because the wheat germ cell-free system enables us to prepare fusion protein samples with tags designed according to the principle of the analytical method chosen.

Applications to substrate discovery for protease

Genome sequencing projects have succeeded in annotating 461 genes for human proteases. Functional analysis of those proteases, however, has been slow, because it has been difficult to produce them using conventional recombinant protein production systems. This difficulty arises from the way proteases are produced in cells, first as inactive precursors which are then processed into mature molecules. In addition, their cleavage sites are generally unique and specific. It is still difficult to predict their mature structures from the genetic information alone, and therefore one has to determine them biochemically, which is a laborious task. Even after the gene of a mature protease could be constructed, its expression in living cells is difficult because expression itself will result in damage to the host cells. The wheat germ cell-free system, by contrast, allows us to produce proteases in mature forms at low temperatures where the rate of protein digestion by produced proteases is sufficiently low. This is because, as the germination of wheat seeds in cold winter demonstrates, the translational machinery of wheat germ functions even at 4°C (Table 1). This system also works well when we search for mature structures: all we have to do is to run parallel translation reactions with a series of DNA templates truncated at their 5′-open reading frame and find a template that gives a smear protein band on SDS-PAGE.

Identifying protein substrates of an enzyme from genetic information alone is difficult and requires biochemical experiments. As an example, we describe below our search for substrates of the protease caspase-3, and its recent results. We first chose the genes of 411 human kinases. We then constructed their DNA templates, which contained a biotin-ligating sequence and a FLAG-tag to produce reporter tags at N- and C-termini of the proteins, respectively. We synthesized mRNAs and used them as translation templates for protein production. We chose 304 high yield samples and, adding caspase-3, incubated them at 37°C. Although we could produce caspase-3 in an active form in the wheat cell-free system, we used for our experiment a commercially available grade made by the *E. coli in vivo* recombinant system, so that we could compare the results of our experiment with previous reports on a consistent basis. We performed the first round of substrate screening with AlphaScreen^{TM} (Streptavidin-coated donor beads, Anti-FLAG-tag IgG-protein A-conjugated acceptor beads), using fluorescence quenching as the indicator of the cleavage of kinase molecular chains. We then took the candidates selected by this preliminary screening, incubated them with caspase-3 and separated the resultant peptide chains by SDS-PAGE. The original N- and C-terminal fragments were detected with Streptavidin-Alexa488 and Anti-FLAG antibody-FITC and, finally, the cleavage site was confirmed by mass spectroscopy. As a result, we identified 43 kinases as candidate substrates of caspase-3 (Sawasaki *et al.*, in preparation), of which 13 were among the 38 caspase-3 substrates that had been known previously and the other 30 were novel

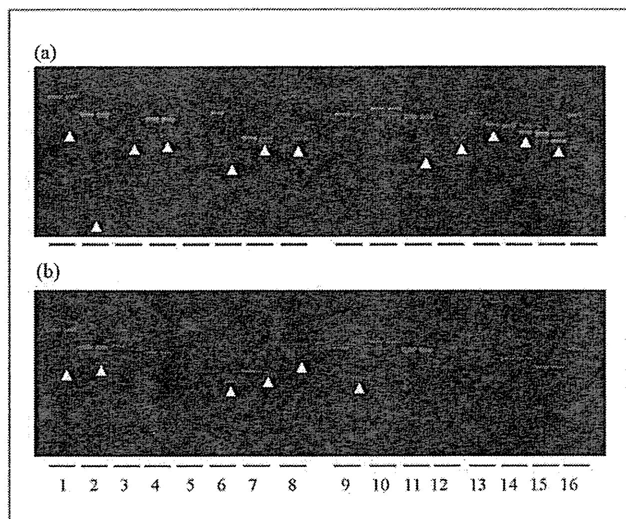

FIGURE 3
Cleavage of substrate kinases by caspase-3. 16 candidates (bars with number) from the first screening using AlphaScreen™ were incubated in the absence (left lane of each substrate underlined) or in the presence (right lane) of caspase-3 for 30 min at 37°C, and were separated by SDS-PAGE. Bands containing the N-terminal **(a)** and C-terminal **(b)** of the original molecules were visualized with Streptavidin-Alexa488 and Anti-FLAG antibody-FITC, respectively. Fragments with molecular weights smaller than 10 kDa were difficult to see. Arrowheads mark the produced fragments.

candidates. This demonstrates the efficacy of *in vitro* screening of substrates, although the results are to be verified directly by cell-level tests.

The second round of screening experiments was carried out as illustrated in Fig. 3. Sixteen candidates out of those identified by the first round of screening using fluorescence were incubated with caspase-3 and separated by SDS-PAGE. Protein or fragment bands with the N-terminal or the C-terminal were then stained and visualized. Although the scope of the experiment was limited to single molecule kinases and did not include complexes with other proteins, we could finally identify 13 new kinases as candidate substrates of caspase-3.

Protein structural analysis

Thanks to its capability to produce large quantities of high quality proteins of both prokaryotic and eukaryotic origin and to the ease of amino acid specific labeling, the wheat germ cell-free protein production system has found application in protein structural analysis. The amino acid metabolic system in wheat germ in hibernation contains only three kinds of transaminase activities, and their inhibitors are well known. With the wheat cell-free system, therefore, protein samples labeled with amino acid residue-specific stable isotopes can be readily prepared in the presence of such inhibitors [22] and used to generate HSQC (heteronuclear single quantum coherence) spectra signals for the location of amino acid residues [23]. The system is also suitable for producing protein samples labeled with SAIL (stereo-array isotope labeling) [24] for the dynamic NMR structural analysis of amino acid residues. In fact, using the wheat germ cell-free system with SAIL amino acids, Hideo Akutsu *et al.* have succeeded in preparing SAIL-labeled membrane proteins in various forms of proteo-liposome for their solid NMR dynamic structural analysis (Akutsu, H., Personal communication, 2010). For the application of the wheat cell-free system to solid NMR analysis, refer to the paper in this issue by Böckmann and colleagues. In X-ray crystallography, the wheat cell-free system facilitates the preparation of selenium-labeled protein samples for MAD (multiwavelength anomalous diffraction) phasing [12]. This is accomplished simply by replacing methionine in the amino acid substrate mixture with selenomehionine. The Center for Eukaryotic Structural Genomics (CESG) at the University of Wisconsin-Madison has successfully adapted the wheat germ cell-free system to build a protein production platform for their NMR and X-ray diffraction structural analyses. Their achievements are presented in their website (http://www.uwstructuralgenomics.org/).

Production of 'difficult-to-express' proteins and their functional and structural analyses

In general, it is difficult to produce a protein complex consisting of hetero subunits in cell-based expression systems. In many cases, it is also difficult to obtain such a complex in an active form in the wheat germ cell-free system, if we synthesize individual subunits separately and then combine them *in vitro* to reconstruct the complex. An easy way to circumvent this difficulty is to co-translate multiple mRNA in the wheat translation solution to produce the protein complex in a folded, active form [25]. An example of recent success is the production of a high quality sample of the human replication protein A complex through co-translation in the wheat germ cell-free system (Makino, S.-I. and Fox, B.G., Personal communication, 2010). This material was subsequently used to produce a crystal, whose structure was then successfully analyzed by X-ray diffraction [26]. Membrane proteins are another group of difficult-to-express proteins. When produced in the wheat germ cell-free system, transmembrane proteins containing single or double transmembrane domains can be recovered in the soluble fraction [13], but those containing multi-transmembrane domains such as GPCR become insoluble aggregates as they are produced. There has been a report of success in producing such a protein in a soluble and active form using the wheat germ cell-free system in the presence of an appropriate surfactant or liposome [27,28].

Conclusion and perspective

Focusing on the functional and structural analyses of eukaryotic proteins, we introduced examples of the application of the wheat germ cell-free protein production system found in recent reports, and some results from our ongoing experiments. We selected those examples to demonstrate the system's characteristics useful for building technology platforms for genome-wide functional and structural analyses.

Rapid advancement in life science calls for the development of certain core technologies for the future, one of which is the protein microarray. The focus of its development would be the stable storage of proteins incorporated in the microarray. It is clear that conventional design concepts developed for DNA microarrays would not work for protein microarrays. This is because, unlike DNA molecules that share stable properties, every protein molecule with its own characteristics requires a specific condition for its stability, and it is difficult to create such conditions in a minute

space on the microarray. It is actually next to impossible to do so, because the functions and properties of a majority of gene products are unknown. It seems that slow progress in the establishment of protein libraries is also attributable to those technical problems yet to be solved. Aiming at solving such problems, we are developing a technique to produce protein microarrays or protein libraries in the fashion of instant noodles. The idea is to store template DNA, mixture of transcription reaction, and the wheat germ translation system in wells of a micro titer plate and freeze-dry them for storage. When we are ready to use proteins, all we have to do is to add water and subject the plate to incubation for a short time to produce fresh proteins *in situ*. Stored in natural lyophilization, the wheat translation enzymes are thought to be stable in their natural state. In fact, we have ascertained through our preliminary experiments that the lyophilized systems do maintain their transcription and translation activities for several years.

We hope that this new wheat germ cell-free protein production system will help to accumulate knowledge about the functions and structures of gene products and accelerate the advancement of life sciences and pharmaceutical R&D.

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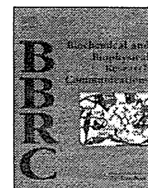

Use of domain enzymes from wheat RNA ligase for *in vitro* preparation of RNA molecules

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ABSTRACT

Wheat RNA ligase can be dissected into three isolated domain enzymes that are responsible for its core ligase, 5′-kinase, and 2′,3′-cyclic phosphate 3′-phosphodiesterase activities, respectively. In the present study, we pursued a practical strategy using the domain enzymes for *in vitro* step-by-step ligation of RNA molecules. As a part of it, we demonstrated that a novel side reaction on 5′-tri/diphosphate RNAs is dependent on ATP, a 2′-phosphate-3′-hydroxyl end, and the ligase domain. Mass spectroscopy and RNA cleavage analyses strongly suggested that it is an adenylylation on the 5′ terminus. The ligase domain enzyme showed a high productivity for any of the possible 16 combinations of terminal bases and a high selectivity for the 5′-phosphate and 2′-phosphate-3′-hydroxyl ends. Two RNA molecules having 5′-hydroxyl and 2′,3′-cyclic monophosphate groups were ligated almost stoichiometrically after separate conversion of respective terminal phosphate states into reactive ones. As the product has the same terminal state as the starting material, the next rounds of ligation are also possible in principle. Thus, we propose a flexible method for *in vitro* RNA ligation.

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1. Introduction

In vitro RNA manipulation with cutting and joining of RNA molecules of defined nucleotide sequences has been of limited use in molecular biology in spite of the important roles of RNA molecules in gene expression. One obstacle to routine RNA manipulation has been the general low yields of RNA ligation. Since the pioneering works in which roles of structural elements in natural RNA molecules were analyzed through substitution with other oligonucleotides [1–5], T4 RNA ligase 1 has been most widely used for this purpose. This enzyme can result in low product yields for some substrates due primarily to the biased preference for terminal bases [6,7] and also to the preference of the enzyme to the substrates with a tRNA fold [8].

Possible alternative RNA ligases that are well characterized are yeast tRNA ligase [9] and plant RNA ligases [10,11]. These eukaryotic RNA ligases have the activities to modify terminal phosphate states of the substrate RNA molecules in addition to the core ligase activity in a single polypeptide. As a result, these enzymes can connect either a 5′-hydroxyl or a 5′-monophosphate end to either a 2′,3′-cyclic monophosphate or a 2′-monophosphate end [12,13], whereas 3′-monophosphate and 2′,3′-dihydroxyl ends are not utilized as a substrate [14]. While yeast tRNA ligase is specific for tRNA precursors [9], plant RNA ligases from wheat and *Arabidopsis thaliana* act on various RNA molecules with single-stranded regions [15–18]. Thus, it seemed to us that the plant enzymes could be more useful for general RNA ligation experiments. We have succeeded in dissecting the wheat enzyme into three independent, non-overlapping, fully active domain enzymes [19]. The ligase (L) domain adenylylates the 5′-phosphate terminus of the substrate RNA and links this terminus to the 3′-hydroxyl end with 2′-phosphate to form a 3′-5′ phosphodiester bond. The kinase (K) domain phophorylates 5′-hydroxyl ends. The 2′,3′-cyclic phosphate 3′-phosphodiesterase (P or CPD) domain opens a 2′,3′-cyclic monophosphate ring to leave a 2′-monophosphate group. The activity of each domain enzyme in the isolated form is at least as high as the activity that is embedded in the full-length enzyme. Because the L enzyme catalyzes ligation between a 5′-phosphate end and a 2′-phosphate-3′-hydroxyl end and probably does not utilize a 5′-hydroxyl end nor a 2′,3′-cyclic monophosphate end, it is expected that the enzyme is useful for *in vitro* RNA preparation through step-by-step ligation of smaller RNA molecules into a larger one with controlling the terminal phosphate states with the aid of end-modifying enzymes.

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In the present study, we investigated the L domain enzymes in more detail on their properties related to the practical usage in *in vitro* RNA ligation. We first characterized an unknown side reaction that was found previously to occur during the ligation reaction of an RNA molecule with a 5′-triphosphate terminus [19]. We then performed a systematic analysis of substrate specificity of the full-length and L domain enzymes. We propose a general RNA ligation scheme that integrates the use of the L domain enzyme, in which a 2′,3′-cyclic phosphate group can serve to block unwanted ligation to the end and can be converted on demand into an active end with the P domain enzyme. The form of the 5′ terminus can also be controlled with commercially available enzymes. The method fits well with the general methods for preparation of RNA molecules with a defined nucleotide sequence that generate 2′,3′-cyclic monophosphate ends.

2. Materials and methods

2.1. Preparation of enzymes and substrate RNAs

The full-length wheat RNA ligase and its domain enzymes were prepared as in the previous study [19]. The domain symbols may be used in this paper to denote these enzymes: "F" stands for the full-length enzyme, "L" for the core ligase domain enzyme, "K" for the kinase domain enzyme, "P" or "CPD" for the phosphodiesterase domain enzyme, and "KP" for the domain enzyme with the kinase and phosphodiesterase activity. DNA enzymes [20] shown in Fig. 1C were oligonucleotides from Invitrogen, which were further purified by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.

The methods for RNA preparation were essentially the same as in the previous study [19], RNAs were synthesized by *in vitro* transcription of DNA templates containing an SP6 promoter by SP6 RNA polymerase (Promega). The template DNAs were prepared by PCR with KOD -Plus- DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) and a set of template oligonucleotides. After transcription, RNAs were separated on 8 M urea-polyacrylamide (acrylamide:bisacrylamide = 19:1) denaturing gels, and were extracted from the gels. The names and the sequences of the starting RNA substrates are as follows: 32NT (pppGAA GAG CUU GCA CUU GGA AAC GUG AUC ACG CA>p, 32 nts); 18NTG, 18NTA, 18NTC, 18NTU (pppGAA GAU GUC ACA UAC CA*N*>p, *N* = G, A, C, and U, respectively, 18 nts); GP1, AP1, CP1, UP1 (*N*AA UAC ACG GAA UUC GAG CUC GUU UUU A, *N* = G, A, C, and U, respectively, 28 nts); and 12NT (pppGAA GAA GAA GAA>p, 12 nts); in which "ppp" denotes 5′-triphosphate, and ">p" denotes 2′,3′-cyclic phosphate. "18NTN" and "NP1" represent any one of the four 18-nucleotide and 28-nucleotide RNA, respectively. The 2′,3′-cyclic phosphate ends of the 12NT and 18NTN RNAs were generated by a *cis*-cleavage of an HDV ribozyme [21] attached to their 3′-ends. The NP1 RNAs have a 5′-hydroxyl end generated by a *cis*-cleavage of a hammerhead ribozyme [22] attached to their 5′-ends. tRNA first half was as described previously [17]. The diphosphate version of 12NT (ppGAA GAA GAA GAA>p, where "pp" represents 5′-diphosphate) was prepared by transcription with 1 mM NTP and 8 mM GDP.

Derivatives of the RNA molecules were prepared by modification of the termini by treatment with one or two of the following enzymes: calf intestine alkaline phosphatase (CIP) (Takara Bio), which removes phosphomonoester, T4 polynucleotide kinase (PNK) (Takara Bio), which phosphorylates the 5′-hydroxyl end to form a 5′-phosphate and also dephosphorylates 2′,3′-cyclic phosphate at the 3′-end to form a 2′,3′-dihydroxyl end, the P enzyme (CPD) [19], which hydrolyzes the 2′,3′-cyclic phosphate to form a 2′-phosphate-3′-hydroxyl end, T4 polynucleotide kinase *pseT1* mutant (*pse*) (Roche) [29], which phosphorylates the 5′-hydroxyl end but does not affect the 3′ end, and the KP enzyme (KPD) [19], which has the 5′-kinase activity in addition to the activity of the P enzyme. These derivatives are denoted in this paper by the RNA name followed by the enzyme symbol(s) shown above in parentheses,

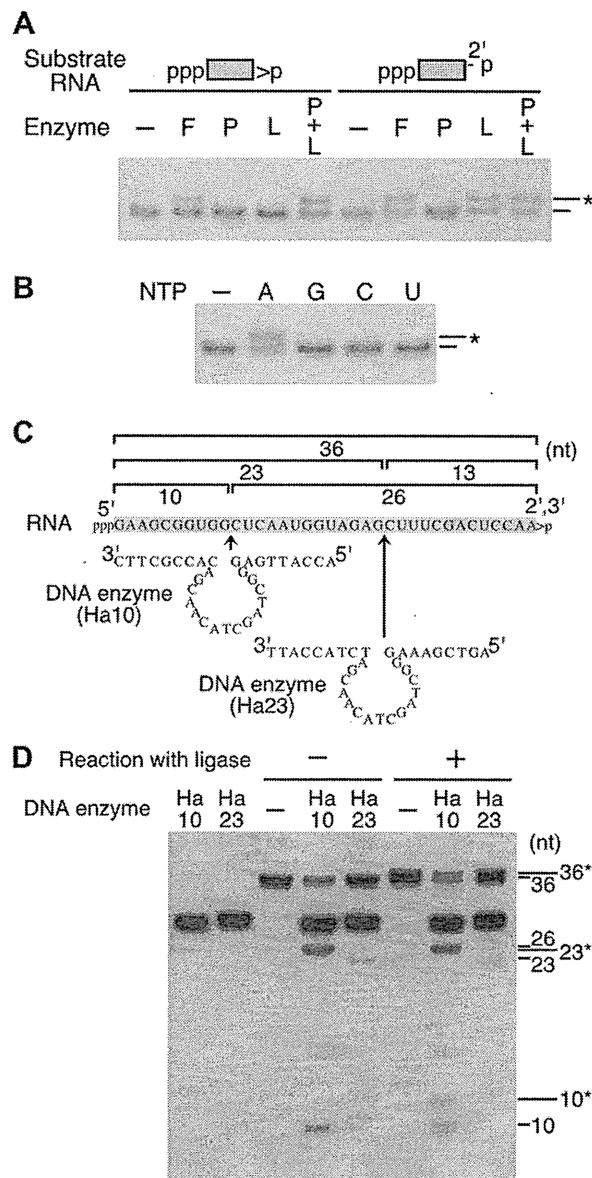

Fig. 1. Analyses of the RNA ligase side reaction. (**A**) A polyacrylamide gel showing that the side reaction is attributed to the ligase domain. The 32NT (left) and 32NTCPD (right) RNAs (3 μM) were incubated with the indicated enzymes at the concentration of 0.86 μM (F), 1.83 μM (P), or 2.1 μM (L), and were separated on the gel. The bands for the side reaction product are indicated by an asterisk. (**B**) A gel showing that ATP, but not the other three nucleoside triphosphates, is required for the side reaction. The 32NT RNA was incubated with the indicated nucleoside triphosphate (1 mM), 1.83 μM P, and 2.1 μM L, and was separated on the gel. (**C**) DNA enzymes [20] and their cleavage sites on the sequence of a target synthetic RNA named previously as tRNA first half [17]. The lengths of the expected RNAs are shown above the RNA sequences. (**D**) A gel separating the RNA products of the DNA enzyme cleavage performed after the side reaction on tRNA first half in order to locate the point of the reaction. 3.4 μM tRNA first half was first incubated with the L and P enzymes and was then incubated with one of the DNA enzymes (8 μM) at 37 °C for 60 min in a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$. The bands assigned to have the side reaction point are indicated by asterisks. The band for the 13-nt fragment from the reaction with Ha23 was not visible because the reaction was not very efficient with this DNA enzyme.

such as 18NTNCIPCPD, which represents the product of 18NTN treated first with CIP and then with CPD. After the treatment with the enzyme(s), the RNAs were purified with phenol/chloroform extraction, ethanol precipitation, and with the gel separation/extraction method when required. Concentrations of the purified RNAs were quantified by measuring the absorbance at 260 nm.

2.2. Enzymatic reactions

Reactions other than the DNA enzyme reaction were performed in a buffer containing 20 mM HEPES-KOH (pH 7.8), 250 mM NaCl, 80 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.01%(w/v) BSA, and, for the kinase and ligase reactions, 1 mM ATP or another nucleotide triphosphate was also added. The reactions were incubated at 26 °C for 120 min, stopped by adding an equal volume of 2× RNA denaturation buffer containing 97%(v/v) formamide, 10 mM EDTA (pH 8.0), 0.015%(w/v) bromophenol blue, and 0.015%(w/v) xylene cyanol FF, heated at 65 °C for 2 min, quickly chilled on ice, and electrophoresed on an 8 M urea-20%(w/v) polyacrylamide gel with 1× TBE buffer. The gels were stained with methylene blue for detection of RNA bands. For quantitation of the RNA bands, NIH Image 1.62 was used, in which the bands of standard RNAs separated on the same gel were used for calibration.

2.3. MS analysis

The MALDI-TOF analyses of RNAs were carried out with a Voyager DE-PRO (Applied Biosystems). Samples were deionized with ZipTip_{C18} (Millipore) using 0.1 M triethylammonium acetate buffer, and eluted with 50 mg/ml 3-hydroxy-2-picolinic acid, 5 mg/ml ammonium citrate in 50%(v/v) acetonitrile. Mass spectra were obtained in the negative ion mode at an acceleration voltage of 25 kV with a delay time at 300 ns. Typically 100 shots were combined for one spectrum. Reflector mode was chosen for analysis below 10 kDa, otherwise analysis was performed by linear mode. Mass calibration was performed with synthesized oligodeoxyribonucleotides.

3. Results

3.1. Analysis of the side reaction on 5′-triphosphate RNA substrates

Use of different terminal phosphate states during *in vitro* intermolecular RNA ligation is a general strategy for controlling which ends to be ligated with each other. We have recognized that the introduction of the 5′-triphosphate group to the RNA substrate intended to be the phosphate acceptor is useful for limiting the 5′ reaction to the other RNA substrate, in part because 5′-triphosphate ends can easily be generated by *in vitro* transcription. While the 5′-triphosphate RNA did not participate in self-ligation, an unexpected reaction was found to take place giving a recognizable band shift in the electrophoresis as mentioned in the previous study [19]. Thus, we characterized this side reaction (Fig. 1).

When a 5′-triphosphate-2′,3′-cyclic phosphate RNA was used as the substrate, the extra band was seen in the resulting mixture with the F enzyme or with both the L and the P enzymes, but not seen in the case of the individual domains (Fig. 1A). If the substrate RNA was pretreated with the P enzyme to make a 2′-phosphate-3′-hydroxyl terminus, the L enzyme was solely sufficient to catalyze the secondary reaction (Fig. 1A). The reaction was dependent on ATP, which could not be replaced by GTP, CTP, or UTP (Fig. 1B). In order to determine which end, 5′ or 3′, of the substrate RNA is modified, we cleaved the RNA site-specifically by DNA enzymes (Fig. 1C) [20]. This revealed that the modification was on the 5′ part of the RNA (Fig. 1D).

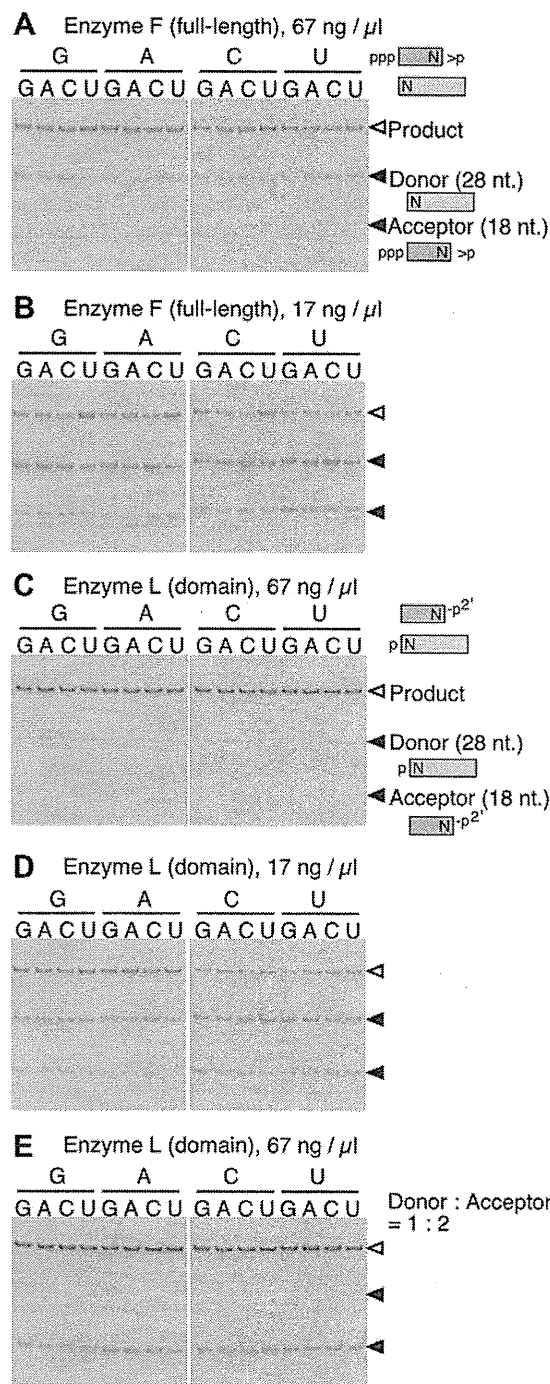

Fig. 2. Terminal base preferences of the full-length and ligase domain enzymes. Polyacrylamide gels resolving the ligation mixtures resulting from the substrates with different terminal nucleotide combinations are shown. A phosphate acceptor substrate (illustrated with an orange box) with the indicated 3′ terminal nucleotide and a phosphate donor substrate (green) with the shown 5′ terminal nucleotide were incubated with the F or L enzyme and separated on the gels. The enzyme used and its concentration, the terminal nucleotides in the acceptor molecules, and the terminal nucleotides in the donor molecules are shown from the top of each panel. The substrate RNAs were 18NTN and NP1 for the F enzyme (**A** and **B**) and 18NTNCIPCPD and NP1PNK for the L enzyme (**C**, **D**, and **E**). The initial concentration of the substrates was 2 μM except for 4 μM 18NTNCIPCPD in **E**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The change in the molecular mass of the RNA was measured to be an increase of 328 Da from the substrate to the product by a

TOF-MS analysis (Fig. S1 in Supplementary material), which coincides with the change for adenylylation. The same phenomenon was seen in the case of a 5′-diphosphate-2′-phosphate-3′-hydroxyl RNA (Fig. S2).

From the above results, the side reaction is likely to be a 5′ adenylylation reaction specific for 5′-triphosphate or 5′-diphosphate RNAs with a 2′-phosphate-3′-hydroxyl end. The ligase domain was able to carry out the side reaction, which seems to proceed by the same mechanism as that of the formation of the 5′ adenylylated intermediate for ligation.

3.2. Terminal nucleotide specificity in the ligation reactions

Although wheat RNA ligase is considered to react with various substrates, there had been no systematic evaluation of its substrate specificity. We executed a systematic analysis by measuring the intermolecular ligation products resulting from the substrates with all the possible combinations of terminal bases (Fig. 2 and Table 1). Although there were slight preferences among the 16 combinations, all of the tested substrates were ligated efficiently. The slightly different pattern between the full-length RNA ligase and the L domain enzyme observed may be due to the altered specificity of the end-modifying activities in the full-length protein. When we used a twofold molar excess of the acceptor over the donor substrate, more than 90 percent of the donor substrate was utilized in the reaction for any combination of the terminal nucleotides (Table 1).

3.3. Terminal phosphate specificity of the L domain enzyme

We next examined the terminal phosphate specificity of the L enzyme. We prepared four substrate RNAs that have an identical nucleotide sequence but differ in the combination of the 5′ and 2′/3′ terminal structures, and investigated whether self-ligation of these substrates are catalyzed by the L enzyme (Fig. 3). In contrast to the full-length enzyme which resulted in self-ligation of all four substrates, the L domain enzyme exclusively ligated the 5′-phosphate-2′-phosphate-3′-hydroxyl RNA (Fig. 3). An efficient intermolecular ligation was also achieved between the 5′-phosphate end and the 2′-phosphate-3′-hydroxyl end (Fig. 3). It is now confirmed that the ligase domain enzyme has a more limited specificity concerning the terminal phosphate states as compared to the full-length protein.

4. Discussion

In vitro RNA cutting and joining makes it possible to prepare hybrid RNA molecules comprising of natural and synthetic molecules that may be useful for biotechnology and for probing the molecular mechanisms involving the RNA molecules at the nucleotide and atomic resolutions. The natural parts may have post-transcriptionally modified nucleotides that may be required for its function. The synthetic parts can be prepared either through chemical methods or enzymatic methods, and they can also have modified or labeled nucleotides. Specific positions of RNA molecules can be cut with ribozymes and DNA enzymes. In our experience, fusion to a hammerhead ribozyme and an HDV ribozyme during *in vitro* transcription is useful for generating a specific 5′-hydroxyl and 2′,3′-cyclic phosphate ends of the transcript. In fact, we adopted this method for preparation of substrate RNA molecules for the assays of the enzymes. DNA enzymes may also be useful for cleaving a specific position to generate the 5′-hydroxyl and 2′,3′-cyclic phosphate ends. In spite of these techniques for preparation of the parts of the hybrid RNA molecules, the methods for joining the parts have so far depended on inefficient ligases.

We observed that the ligase domain catalyzes a reaction that is likely to be an adenylylation of 5′-tri- or diphosphate termini. This reaction is likely a side reaction accompanying the formation of the 5′-adenylylated intermediate during ligation. Both reactions are dependent on the 2′-phosphate and 3′-hydroxyl groups in the substrate RNA. When a 5′-monophosphate terminus is adenylylated as a ligation intermediate, it will be subsequently used for the ligation reaction with a 2′-phosphate-3′-hydroxyl RNA terminus, whereas adenylylated products of 5′-tri- or diphosphate termini will not participate in the ligation reaction. It is now very likely that the hydrolysis of the cyclic phosphate precedes the adenylylation on the 5′ phosphate by the native full-length enzyme, and that the L domain itself is responsible for the specificity to the 2′-phosphate-3′-hydroxyl ends. Similar adenylylation side reactions on nucleoside 5′-tri- or diphosphate are seen in other ligases that share conserved motifs with wheat RNA ligase, including T4 DNA ligase [23] and T4 RNA ligase [24]. These ligases have homology to guanylyltransferase, which guanylylates the 5′-diphosphate ter-

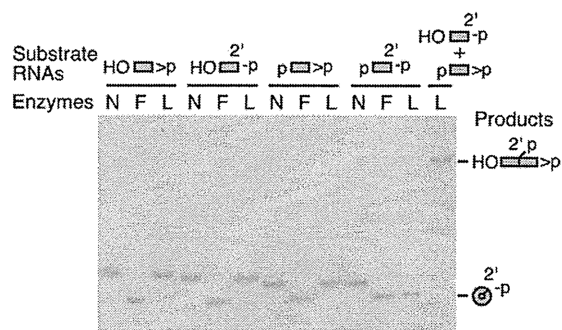

Fig. 3. Selectivity of the ligase domain enzyme for the 5′-phosphate and 2′-phosphate-3′-hydroxyl ends. A gel separating the resulting RNAs from the substrates with different terminal structures is shown. The RNA substrates, shown schematically on the top, were incubated with nothing (N) or with the indicated enzyme at 0.86 μM (F) or 1.05 μM (L) and were analyzed. 4 μM of 12NTCIP, 12NTCIPCPD, 12NTCIP*pse*, or 12NTCIPKPD, or 2 μM each of 12NTCIPCPD and 12NTCIP*pse* were used. Ligated products are schematically shown on the right.

Table 1
Ligation efficiencies for different substrates.

Enzymes	Enzyme concentrations (μM)	Substrate RNAs (μM)		Conversion (%)															
		Acceptor		G				A				C				U			
			Donor	G	A	C	U	G	A	C	U	G	A	C	U	G	A	C	U
F	0.57	2	2	59	63	59	68	59	65	56	67	60	64	64	67	53	55	50	56
	0.14	2	2	32	33	27	50	35	40	25	51	35	34	27	44	22	23	18	33
L	0.70	2	2	79	72	77	76	78	80	86	83	81	73	81	78	80	75	79	83
	0.18	2	2	47	46	42	55	51	58	48	60	25	30	27	34	23	24	28	31
	0.70	4	2	>90, in any combination															

minus of mRNA precursors to form a cap structure [25]. It is therefore conceivable that these ligases also have the capacity for modifying the 5′-tri- and diphosphate termini. Whether this side reaction plays any role in cellular system remains to be investigated.

We also confirmed that the F and L enzymes have broad substrate sequence specificities (Fig. 2 and Table 1). It is well known that ligation reactions with T4 RNA ligase may be inefficient depending on the nucleotides around the ligation site [6,7]. The dependence of the efficiency on the substrate sequences has been systematically investigated, and the yields could be in some cases less than several percents [7]. Although a method for avoiding very low yields have been devised [26] in which the yield is around 50%, the method still requires design of a splint DNA molecule on a case-by-case basis. T4 DNA ligase is also used for splinted ligation of RNA molecules at a double-stranded region, while this method requires very large amounts of the enzyme [27,28]. Although we have observed some difficulty in the ligation of structured RNAs with our F and L enzymes (unpublished data), these enzymes have a desirable sequence-specificity for general ligation.

The data in Fig. 3 confirm the specificity of the L enzyme for the 2′-phosphate-3′-hydroxyl ends and demonstrate that the ends to be joined can be controlled strictly. We propose a flexible method for joining RNAs with defined sequences facilitated by the use of the domain enzymes, in which, once RNA units having a 5′-hydroxyl and a 2′,3′-cyclic phosphate ends are prepared, they can be linked together specifically in any order with a good yield. The starting RNA units can be easily prepared with the use of ribozymes and/or DNA enzymes. RNase T1 digests of natural RNAs may also be useful. The 3′ molecule to be joined at the 5′-end can be activated through the reaction catalyzed either by the K enzyme or the *pseT1* mutant of T4 polynucleotide kinase [29]. The 5′ molecule to be joined at the 3′-end can be converted to have 5′-hydroxyl, 2′-phosphate ends with the P enzyme. The two substrates can now be joined specifically by the L enzyme to generate a new RNA molecule with 5′-hydroxyl and 2′,3′-cyclic phosphate ends. Both substrates and the product neither self-ligate nor concatenate. Although the 2′-phosphate group at the ligation junction remains after the reaction, this issue can be resolved by using a 2′-phosphotransferase, an enzyme playing a role in the splicing of tRNA [30]. The yeast Tpt1 enzyme has already been used for removing the 2′-phosphate group of a wide variety of substrates [14]. We also have tested this enzyme with a substrate RNA and have obtained a good result. The strategy is advantageous also in that the ligation product has the same terminal state as the starting RNA units, because some applications of *in vitro* RNA manipulation require two or more consecutive rounds of ligation. Further investigation may be necessary for establishing the versatility of the ligation scheme with these enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.108.

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Short Communication

Ca^{2+}-dependent protein kinases and their substrate HsfB2a are differently involved in the heat response signaling pathway in Arabidopsis

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Abstract Little is known about the mechanisms by which Ca^{2+}-binding sensory proteins direct the plant heat shock (HS) response. Since two Ca^{2+}-dependent protein kinases (CPK3 and CPK13) were recently shown to phosphorylate the heat shock transcription factor HsfB2a, we assessed in the current study whether these kinases are also involved in HS signal transduction, by monitoring the transcriptional profile of HS protein (Hsp) family genes in Arabidopsis Col-0 plants (WT) and the corresponding mutants. Both with and without HS, the gene transcript levels of *Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI* were found to be lower in *cpk3* and *cpk13* mutants compared to WT, resulting in the impairment of basal thermotolerance in the mutants. To determine the *in vivo* function of CPKs, CPK3/13 and their substrate HsfB2a (heat shock transcription factor) were co-expressed as cofactors for the transient expression of a reporter (GUS) gene under the control of heat shock element (HSE) in *Nicotiana benthamiana* leaves. However, CPK3/13-phosphorylated HsfB2a did not function in the suppression/activation of HSE-promoted expression in the transient expression system. Implications for possible signal trafficking via CPKs and Hsfs are discussed.

Key words: Arabidopsis, Ca^{2+}-dependent protein kinase (CPK), heat shock response, HsfB2a.

Abiotic stresses, such as drought, salinity or extreme temperatures, are serious threats to agriculture and result in deterioration of the environment. Elucidating the various mechanisms of plant responses to stress and their roles in acquired stress tolerance is thus of great practical and basic importance (Wang et al. 2004). Various stress effects result in the appearance of partly denatured proteins in cells that activate a stress response system. Many molecular chaperones are stress proteins and many of them were originally identified as heat shock (HS) proteins (Hsp) (Wang et al. 2004). Most Hsps begin to be expressed as a result of the HS-induced trimerization of an HS transcription factor (Hsf), which enables Hsf to bind to the HS element (HSE) in the promoter region of Hsp genes (Baniwal et al. 2004; von Koskull-Döring et al. 2007), eventually resulting in the potential acquisition of thermotolerance by plants (Montero-Barrientos et al. 2010).

Plants possess several classes of Ca^{2+}-binding sensory proteins, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins, and Ca^{2+}-dependent protein kinases (CPKs) (Sanders et al. 2002). Several Ca^{2+}-binding sensory proteins [e.g., Ca^{2+}/calmodulin-binding protein kinase (AtCBK3), a member of the PPP family (AtPP7)], are known to play roles in heat-shock signal transduction in which Hsf is phosphorylated in both constitutive and HS-induced manners (Li et al. 2004; Liu et al. 2005; Liu et al. 2007; Liu et al. 2008). Moreover, the CPKs are of special interest, since they represent a novel class of Ca^{2+} sensors, having both a protein kinase domain and a calmodulin-like domain (including an EF-hand calcium-binding site) in a single polypeptide (Klimecka and Muszyńska 2007). CPKs constitute a large family of serine/threonine protein kinases that are broadly distributed in the plant kingdom. In a previous study we demonstrated with *in vitro* kinase assays that

Abbreviations: CPK, Ca^{2+}-dependent protein kinase; HS, heat shock; HSE, heat shock element; Hsf, heat shock factor; Hsp, heat shock protein

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CPK3 and CPK13 proteins phosphorylate the heat shock transcription factor HsfB2a and with *in vivo* agroinfiltration assays that the CPK-mediated phosphorylation of HsfB2a promotes the transcriptional activation of the plant defensin gene *PDF1.2* in a defense response (Nagamangala Kanchiswamy et al. 2010). Moreover, CPK3 kinase activity appeared to be induced by heat stress treatment (Mehlmer et al. 2010). These results prompted us to assess whether the CPK3 and CPK13 cascades are also involved in HS signal transduction. The expression of various Hsp family genes (*Hsp70*, *Hsp101*, *Hsp17.4-CIII* and *Hsp15.7-CI*) was assayed by quantitative reverse transcription (RT)-PCR in Arabidopsis Col-0 plants (WT) and two T-DNA insertion lines: *cpk3* (SALK_022862) and *cpk13* (SALK_057893) that were subjected to heat stress treatment at 40°C for 3 h. Disruption of CPK mRNA expression in leaves of the respective *cpk* mutants was confirmed previously (Nagamangala Kanchiswamy et al. 2010). Compared to the levels in WT seedlings, unheated *cpk3* seedlings contained significantly lower transcript levels for the *Hsp101* and *Hsp15.7-CI* genes, whereas *cpk13* seedlings contained low transcript levels for all of the Hsp genes analyzed (Figure 1A). In both T-DNA insertion lines, however, no visible phenotypical changes were observed in the unheated condition (data not shown).

WT plants reacted to HS by increasing the transcript levels of all Hsps, compared to the respective levels in unheated plants. In contrast, the transcript levels in *cpk3* and *cpk13* plants were significantly lower compared to those in WT plants, with the sole exception of *Hsp15.7-CI* expression in *cpk3* seedlings (Figure 1B). Both *cpk3* and *cpk13* seedlings were impaired, compared to WT, in thermal tolerance at 40°C for 3 h (Figure 1C). Genetic analysis has shown that Hsp101 is absolutely essential for thermal tolerance in Arabidopsis (Tonsor et al. 2008), and this Hsp has been reported to play a role in preventing oxidative stress (Zhang et al. 2009). Hsp70 is one of the major classes of chaperone molecules and is involved in a variety of tasks in eukaryotic cells, and increased Hsp70 synthesis results in a marked increase in stress tolerance (Hu et al. 2010; Montero-Barrientos et al. 2010). Although plants generate an array of high molecular weight Hsps, most of the translation capacity is devoted to the synthesis of the small Hsps (e.g., *Hsp15.7-CI* and *Hsp17.4-CIII*) which also play an important role in the acquisition of thermal tolerance (Yildiz and Terzi 2008). Accordingly, the drastically decreased levels of expression of Hsps in *cpk3* and *cpk13* seedlings, especially, with HS treatment, very likely caused impairment of the plants' basal thermotolerance.

As described above, it was found that CPK3 and CPK13 phosphorylate HsfB2a (heat shock transcription factor) (Nagamangala Kanchiswamy et al. 2010). This

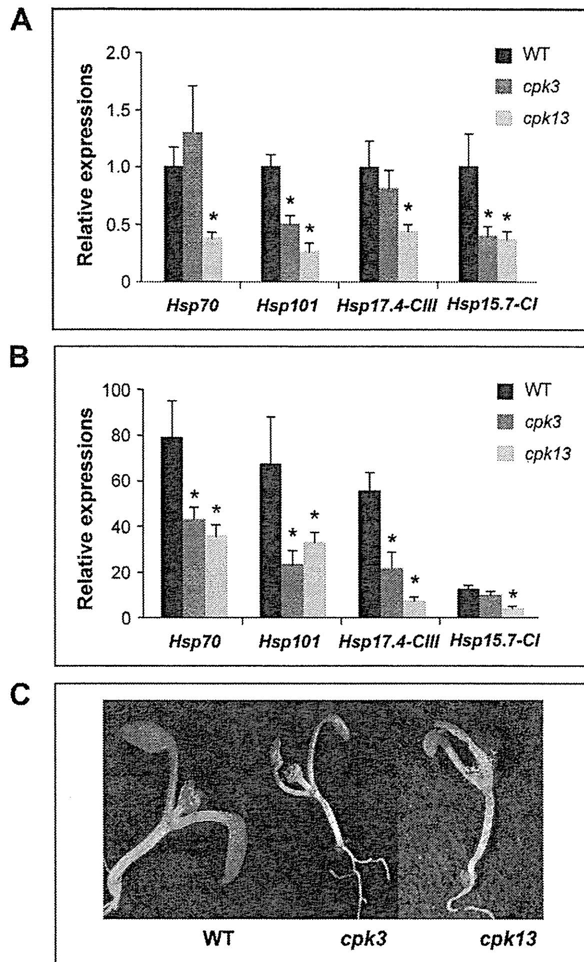

Figure 1. Effects of loss of CPK function on heat response signaling pathway in Arabidopsis seedlings. Arabidopsis seedlings (Col-0) were grown on MS medium containing 2% sucrose and 0.8% agarose in a growth chamber at 22°C. Transcript levels of Hsp genes in seven-day-old seedlings of WT, *cpk3* and *cpk13* were assessed before (A) and after (B) heat stress treatment at 40°C for 3 h. Quantitative reverse transcription (RT)-PCR was done on an ABI Prism® 7000 Sequence Detection System (Applied Biosystems). Primers used for this study are shown in Table 1. Transcript levels of genes were normalized by those of *ACT1* (At2G37620) measured in the samples and expressed relative to the normalized transcript levels in the leaves of unheated WT plants. Data represent the mean+SE (n>5). An asterisk (*) indicates that the mutant was significantly different from WT for the indicated gene and treatment (P<0.05, ANOVA). (C) Impaired thermal tolerance at 40°C for 3 h both *cpk3* and *cpk13* seedlings with respect to compared to WT phenotype is shown.

Hsf appears to be induced by HS treatment (Li et al. 2009), and belongs to the Hsf class B transcription factors (B-Hsfs). The function of class B-Hsfs differs from that of class A-Hsfs due to a structural variation within the oligomerization domain and the lack of an AHA-motif, which is required for the transcriptional activation function of class A-Hsfs (von Koskull-Döring et al. 2007). Since class B-Hsfs have the capacity to bind to similar or the same sites in the heat shock gene promoters as class A-Hsfs, most of them may act as

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