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TRAP Display: A High-Speed Selection Method for the Generation of Functional Polypeptides

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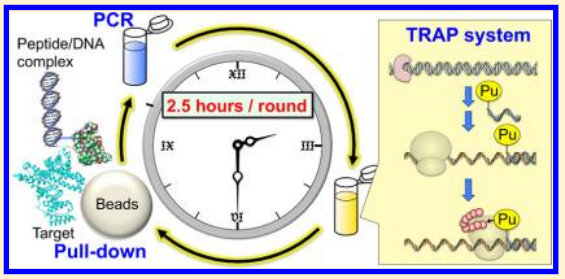
Supporting Information

ABSTRACT: Here, we describe a novel method that enables high-speed *in vitro* selection of functional peptides, peptidomimetics, and proteins via a simple procedure. We first developed a new cell-free translation system, the TRAP system (transcription−translation coupled with association of puromycin linker), which automatically produces a polypeptide library through a series of sequential reactions: transcription, association of puromycin-DNA linker, translation, and conjugation between the nascent polypeptide and puromycin-DNA linker. We then applied the TRAP system for the selection of macrocyclic peptides against human serum albumin. Six rounds of selection using TRAP display were performed in approximately 14 h, yielding macrocyclic peptides with nanomolar affinity to their target protein. Because TRAP display enables high-speed selection of functional polypeptides, it will facilitate the generation of various polypeptides that are useful for biological and therapeutic applications.



INTRODUCTION

In vitro display methods have been used to generate various functional peptides, peptidomimetics, and proteins. Success in *in vitro* selection of polypeptides with desired properties require one-to-one correspondence between the genotype (DNA or mRNA) and phenotype (encoded polypeptide), maintained by a physical linkage. Formation of such a physical linkage determines the performance of the selection method, such as (1) time and effort to complete the selection, (2) availability of selective conditions that need to be precisely designed to select polypeptides with desired properties, and (3) the variety of amino acids that can be used as building blocks of the polypeptide libraries.

For example, ribosome display can rapidly prepare the peptidyl-tRNA/mRNA/ribosome complexes from the corresponding DNAs because the linkages mediated by the ribosome and peptidyl-tRNA can be automatically formed in a coupled transcription−translation system.[1,2] However, ribosome display selection may suffer from unpredictable interactions between the displayed polypeptide and the very large ribosome.[3] Moreover, selection steps must be performed before reverse transcription (RT) and under a high magnesium concentration to avoid disassembly of the peptidyl-tRNA/mRNA/ribosome complexes.[1,2,4] Such conditions can potentially produce not only desired polypeptide binders but also undesired RNA aptamers[5] because RNA folding is generally stabilized by magnesium ions. Similarly, display technologies using template DNA-binding anchor proteins (streptavidin or DNA replication initiator protein, RepA) as the linkage also allow automatic formation of the polypeptide/DNA complexes from the template DNAs in a coupled transcription−translation system.[6,7] Although these protein-based linkages are stable under various conditions, this system cannot be easily applied to the selection of peptidomimetic composed of non-proteinogenic amino acids via genetic code reprogramming[8−11] because these anchor proteins cannot be synthesized in a translation system lacking proteinogenic amino acids.

The mRNA display (or *in vitro* virus) systems circumvent these difficulties by using non-protein-based linkage mediated by a small molecule, puromycin (Pu).[12,13] Puromycin is an analogue of the 3′-end of aminoacyl-tRNA and attacks a nascent polypeptide in the ribosome. Therefore, only the attachment of the puromycin linker to mRNA is required for producing a robust polypeptide/mRNA complex. To date, several attachment methods, such as ligation,[12,13] photo-cross-linking,[14] and hybridization using 2′-*O*-methyl-RNA,[15] have been developed. However, these require multiple stepwise manipulations, including transcription, attachment of the puromycin linker, and translation, to be performed separately because an independent step is required to modify the mRNA with the puromycin linker. These multistep manipulations make mRNA display complicated and time-consuming, typically requiring 2−3 days per round.[16,17] Therefore, there is a need for more simple and convenient display methods that can rapidly prepare polypeptide/mRNA complexes with small linkages from the template DNAs.

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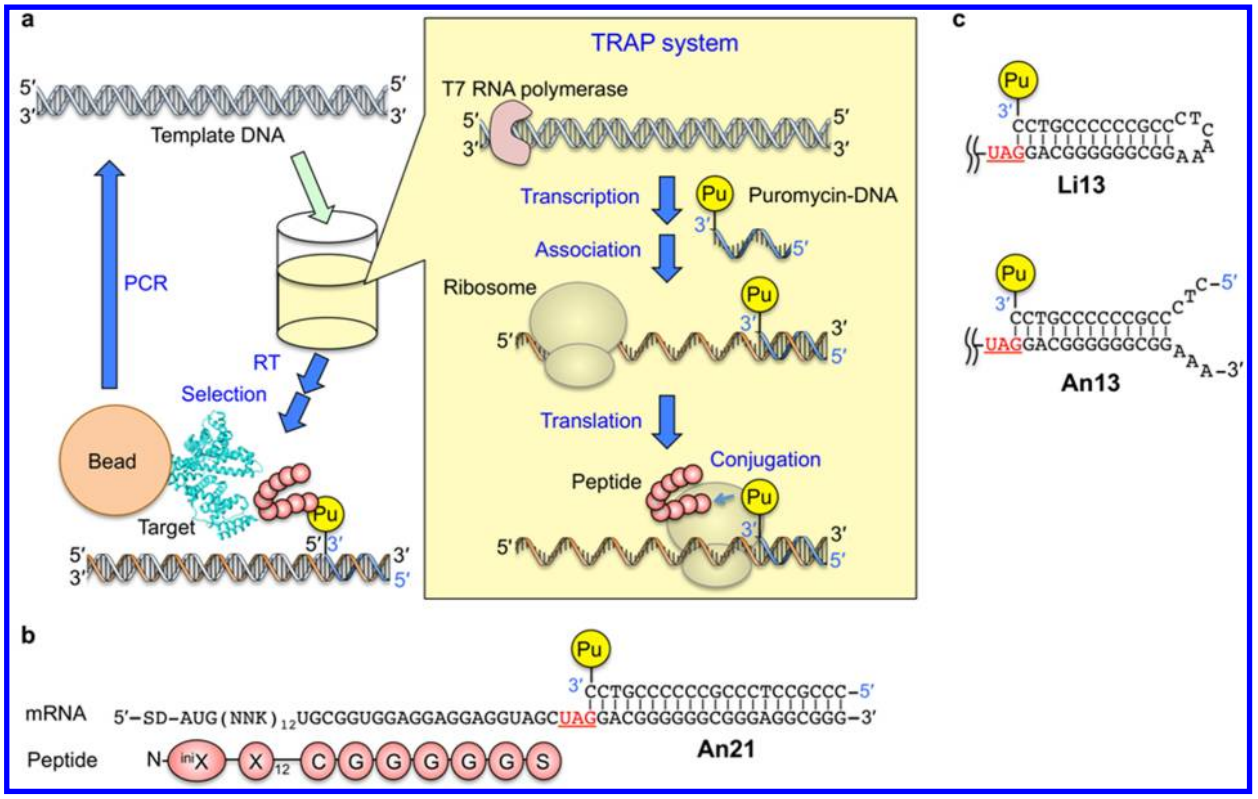

Figure 1. Design of TRAP display. (a) Schematic view of TRAP display selection, which consists of four steps: the polypeptide/mRNA complex formation in the TRAP system, reverse transcription (RT), selection against target-immobilized beads, and PCR. In the TRAP system, transcription, association of a Pu-linker, translation, and conjugation between polypeptide and puromycin are coupled; therefore, the polypeptide/mRNA complexes are automatically formed by simply adding template DNAs to the TRAP system. (b) A random pool of mRNA and the Pu-linker complex used in TRAP display (An21). The Pu-linker is annealed to the 3′-UTR of the mRNA to form a 21-base-pair DNA/RNA duplex. The mRNA sequence for a cysteine residue and the following spacer amino acid residues (Gly-Gly-Gly-Gly-Gly-Ser) and a blank UAG codon (colored in red) are placed after the $(NNK)_{12}$ random sequence. (c) Structure of mRNA/Pu-linker 13-base-pair duplex with ligation (Li13) or without ligation (An13). Abbreviations: Pu-linker, puromycin-DNA linker; UTR, untranslated region.

Here, we describe TRAP (transcription–translation coupled with association of puromycin linker) display, which facilitates high-speed *in vitro* selection of polypeptides and peptidomimetics using a simple procedure (Figure 1a). In the TRAP system, sequential reactions, including transcription of DNA into mRNA, association of a puromycin-DNA linker (Pu-linker), translation of mRNA into polypeptides, and conjugation between the polypeptide and Pu-linker, proceed continuously. A stable polypeptide–Pu-linker/mRNA complex is automatically formed from its template DNA so that the time for preparation of polypeptide libraries displayed on the corresponding mRNA can be markedly reduced. Thus, TRAP display can greatly accelerate the speed of polypeptide selections to typically less than 3 h per round.

In this study, we first compared the stability of peptide–Pu-linker/mRNA complexes and the efficiency of formation of random peptide–Pu-linker/mRNA complexes in a conventional cell-free translation system and in the TRAP system. We then demonstrated enrichment of a model T7-tag peptide (T7-peptide) from a random peptide-encoding pool of DNAs (random-DNAs) spiked with the T7-peptide-encoding DNA (T7-DNA). Using TRAP display, we selected thioether-macrocyclized peptides against human serum albumin (HSA) from a peptide library containing as many as 10^{12}–10^{13} unique sequences. More importantly, six rounds of selection were performed in only about 14 h, demonstrating the high-speed advantage of TRAP display.

EXPERIMENTAL SECTION

Preparation of DNA, mRNA, and Aminoacyl-tRNAs. The preparation of T7-DNA, random-DNA, T7-mRNA, and random-mRNA is described in the Supporting Information. Phe-$tRNA^{Asn\text{-}E2}_{CUA}$ and biotin-Phe-$tRNA^{fMet}_{CAU}$ were prepared according to the previous reports[10] (see also Supporting Information). Synthesis of *N*-[3-(2-chloroacetamide)benzoyl]-L-Phe-CME (ClAB-L-Phe-CME, where CME is cyanomethyl ester) and preparation of ClAB-L-Phe-$tRNA^{fMet}_{CAU}$ are also described in the Supporting Information.

Preparation of a Conventional Cell-Free Translation System and the TRAP System. Creatine kinase, creatine phosphate, and *Escherichia coli* tRNAs were purchased from Roche Diagnostics (Tokyo, Japan). Myokinase was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Ribosome was purified from *E. coli* A19 strain by using a similar procedure reported previously.[18] Chemicals and proteins for translation were prepared by a similar procedure of previously published papers.[9,19−21] The protein concentration in the stock solution B (solB) and the final concentration in the reaction mixture are listed in Table S2. The concentration of tRNAs and other small-molecule factors in the stock solution A (solA) and in the reaction mixture are listed in Table S3.

The reaction mixture of a conventional cell-free translation system was prepared by mixing the solA (11%, v/v) and solB (10%, v/v) and other solutions. The reaction mixture of the TRAP system was prepared by mixing solA (11%, v/v), solC (11%, v/v), Pu-linker (1.1 μM in final concentration), and other solutions (Table S2).

Preparation of the Li13-, An13-, and An21-Type Pu-Linker/mRNA Complex. Preparation of the Li13-type Pu-linker/mRNA complex is described in the Supporting Information. The An13- and An21-type Pu-linker/mRNA complexes were prepared by mixing

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mRNA and 1.1 equiv of Pu-linker. The Pu-linker/mRNA complex concentrations described below were the concentrations of the mRNA.

Analysis of the T7-Peptide−Pu-Linker/mRNA/cDNA Complex Stability. The translation solution was prepared as described above. The translation mixture containing each 0.25 mM 20 proteinogenic amino acid, 0.1 μM Li13-, An13-, or An21-type Pu-linker/T7-mRNA complex, and the corresponding 1 μM Pu-linker/random-mRNA complex was used in this experiment. Alternatively, 25 μM Phe-tRNA$^{Asn\text{-}E2}_{CUA}$ was added to the mixture in order to reassign the UAG codon from blank to Phe.

The reaction was performed as follows. The translation mixture (2 μL) was incubated at 37 °C for 15 min. After addition of 1 μL of 50 mM ethylenediaminetetraacetic acid (EDTA, pH 7.5), the mixture (3 μL) was added to 1 μL of 4× RT mix: 200 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl (pH 8.3), 300 mM KCl, 75 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 2 mM each dNTP (dATP, dTTP, dGTP, dCTP), 10 μM G5S-4.R20, and 6 U ReverTraAce (TOYOBO). The final concentrations of mRNA templates and primer were 0.05 μM T7-mRNA, 0.5 μM random-mRNA, and 2.5 μM G5S-4.R20. The mixture was incubated at 42 °C for 30 min.

T7-peptide pull-down was performed as follows. After 10-fold dilution of the RT mixture with HBST [50 mM 2-ethanesufonic acid (Hepes)-KOH pH 7.5, 300 mM NaCl, 0.05% Tween20], the diluted mixture (10 μL) was mixed with anti-T7-peptide antibody (MBL) immobilized on Dynabeads Protein G (VERITAS) and incubated for 15 min at 4 °C. The beads were washed with 10 μL of HBST three times and were suspended in 25 μL of 0.5× PCR buffer [5 mM Tris-HCl pH 8.4, 25 mM KCl, 0.05%(v/v) Triton X-100]. The solution was heated at 95 °C for 5 min, and 1 μL of the supernatant was added to 19 μL of 1× PCR mix (1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM each dNTP, 0.25 μM T7SD8M2.F44, 0.25 μM G5S-4.R20, and *Taq* DNA polymerase). After PCR was performed (94 °C for 20 s, 60 °C for 20 s, 72 °C for 30 s), the amplified DNA was analyzed by native PAGE.

Quantitation of the Random-Peptide−Pu-Linker/mRNA Complex Formation in the TRAP System. The translation mixture containing each 0.25 mM 19 proteinogenic amino acid (−Met), 25 μM biotin-Phe-tRNA$^{fMet}_{CAU}$ and Li13- or An21-type 1 μM Pu-linker/random-mRNA complex was used in this experiment. Alternatively, 25 μM Phe-tRNA$^{Asn\text{-}E2}_{CUA}$ was added to the mixture in order to reassign the UAG codon from blank to Phe. The reaction mixture of the TRAP system containing each 0.25 mM 19 proteinogenic amino acid (−Met), 25 μM biotin-Phe-tRNA$^{fMet}_{CAU}$ and 5% (v/v) random-DNA RT-PCR solution was also used in this experiment.

Translation (2 μL) and RT (4 μL) were performed as described above. The biotin-random-peptide−Pu-linker/mRNA/cDNA complexes were recovered by using streptavidin (SA) magnetic beads, and the recovered cDNA was quantified by real-time PCR. The reaction mixture containing reverse transcribed random cDNA was serial-diluted and used as standards.

Selective Enrichment of T7-Peptide Using a Conventional mRNA Display and TRAP Display Format. The translation mixture containing each 0.25 mM 20 proteinogenic amino acid, 0.3 nM Li13- or An21-type Pu-linker/T7-mRNA complex, and the corresponding 1 μM Pu-linker/random-mRNA complex was used in this experiment. For the conventional mRNA display format (Li13-type), 25 μM Phe-tRNA$^{Asn\text{-}E2}_{CUA}$ was added to the mixture in order to reassign the UAG codon from blank to Phe. The reaction mixture of the TRAP system containing each 0.25 mM 20 proteinogenic amino acid and 5% (v/v) RT-PCR solution of T7-DNA and random-DNA mixed in a 1:3,000 ratio was also used in this experiment.

Translation (2 μL), RT (4 μL), T7-peptide pull-down assay, and PAGE analysis were similarly performed as described above.

***In Vitro* Selection of Macrocyclic Peptides by Using TRAP Display.** The translation solution was prepared as described above. Translation mixture (50 μL) containing 0.25 mM each 19 proteinogenic amino acid (−Met), 10 μM ClAB-L-Phe-tRNA$^{fMet}_{CAU}$, 1 μM mRNA library and 1.1 μM Pu-linker.an21 was incubated at 37 °C for 15 min. After addition of 12.5 μL of 100 mM EDTA (pH 7.5), the mixture was incubated with HSA-immobilized SA beads (1.6 pmol of HSA) at room temperature for 20 min. The beads were washed with 60 μL of HBST three times and were suspended in 5 μL of 1× RT mix [1 μM G5S-4.R20, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dNTP, 5 U M-MLV (+)(Promega)]. After incubation at 42 °C for 30 min, the mixture was added to 100 μL of 1× PCR mix (1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM each dNTP, 0.25 μM T7SDM2.F44, 0.25 μM G5S-4an21.R41) and was heated at 95 °C for 5 min. A 1 μL aliquot of the mixture was used for quantification of the recovered cDNA, and the rest of the mixture was used for PCR (94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s) after addition of *Taq* DNA polymerase.

The reaction mixture (20 μL) of the TRAP system containing 0.25 mM each 19 proteinogenic amino acid (−Met), 10 μM ClAB-L-Phe-tRNA$^{fMet}_{CAU}$, and 5% (v/v) PCR crude solution was incubated at 37 °C for 15 min. After addition of 5 μL of 100 mM EDTA (pH 7.5), the mixture was added to 8.53 μL of 4× RT mix [200 mM Tris-HCl (pH 8.3), 300 mM KCl, 75 mM $MgCl_2$, 4 mM DTT, 2 mM each dNTP, 10 μM G5S-4.R20, 6 U ReverTraAce], and incubated at 42 °C for 30 min. After the reaction was quenched by adding 6 μL of 100 mM EDTA (pH 7.5) and 4 μL of 500 mM Hepes, the solution was mixed with HSA-immobilized SA beads (1.1 pmol of HSA) and incubated at 25 °C for 10 min. After the beads were washed with 60 μL of HBST three times, the recovered beads were mixed with 40 μL of 1× PCR mix, and the solution was heated at 95 °C for 5 min. Quantification of recovered cDNA by real-time PCR and amplification of the cDNA by PCR followed the same procedure as in the first round.

From the third round, the scale of the reaction mixture was reduced four times. Additionally, negative selection using SA beads was performed three times before the positive selection. In the fifth and sixth rounds, stringent wash with 200 μL of HBST was performed at 37 °C for 30 min at the second wash. After the sixth round, the amplified cDNA was cloned and sequenced.

Binding of Clonal Peptides and Their Derivatives to HSA in Display Format. The mRNA of p**1** and p**2** was synthesized from colony PCR product. Each mRNA of peptides with reverse or shuffled sequence of p**1** and p**2** was prepared as follows. DNA template was prepared using T7SD8M2.F44 as a forward primer and SD8No38r-evG5S4.R69, SD8No38ranG5S4.R69, SD8No41revG5S4.R75, or SD8No41ranG5S4.R75 as a reverse primer. Extension-PCR and transcription were performed using a procedure similar to that used for T7-mRNA preparation. The mRNA was purified by phenol/chloroform extraction and 2-propanol precipitation. The pellet was dissolved in ultrapure water.

Translation (2 μL), RT (4 μL), and the quenching reaction (5.25 μL) were performed as described above. A 2 μL aliquot of the resulting solution was mixed with HSA-immobilized SA beads (0.3 pmol HSA) or ligand-free SA beads. After the incubation at room temperature for 10 min, the beads were washed with 10 μL of HBST three times. At the second wash, stringent wash with 100 μL of HBST was performed at 37 °C for 30 min. Recovered cDNA was quantified by real-time PCR.

RESULTS

Design of the TRAP System. mRNA display requires multiple manipulation steps for preparation of the polypeptide/mRNA complex, which makes this display selection complicated and time-consuming. To prepare the polypeptide/mRNA complexes more rapidly, we designed the novel TRAP coupling system, which is a reconstituted cell-free transcription−translation coupling system containing a Pu-linker and lacking release factor 1 (RF1). In the TRAP system, multiple reactions proceed sequentially in a single step, as shown in Figure 1a. First, template DNA is transcribed to mRNA by T7 RNA polymerase. Then, the transcribed mRNA is "trapped" by the Pu-linker DNA through formation of a stable duplex between the Pu-linker DNA, and the mRNA as a GC-rich sequence complementary to the Pu-linker DNA is placed at the 3′-untranslated region (3′-UTR) of the mRNA. The ribosome

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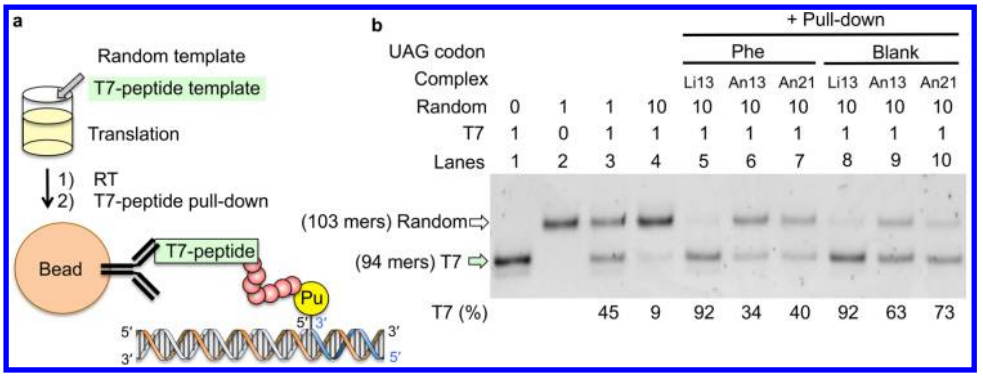

Figure 2. Examination of the peptide–Pu-linker/mRNA complex stability using the T7-peptide pull-down assay. (a) Scheme of the T7-peptide pull-down assay. Mixtures of the T7-peptide and random pool (NNK_{12}) templates were translated. After reverse transcription, the complexes were recovered by anti-T7 antibody-immobilized beads. cDNAs of selected complexes were amplified and analyzed using PAGE. (b) Evaluation of the peptide–Pu-linker/mRNA complex stability through the selection processes. Lanes 1–4 contain DNA markers synthesized from the T7-mRNA, random-mRNA, and a 1:1 or 1:10 mixture of T7- and random-mRNAs. In lanes 5–10, T7- and random-mRNAs were mixed in a 1:10 ratio in the translation system where a UAG codon is reassigned to Phe (lanes 5–7) or a blank (lanes 8–10). The following mRNA templates were used in translation: lanes 5 and 8, Li13-type mRNAs; lanes 6 and 9, An13-type mRNAs; lanes 7 and 10, An21-type mRNAs. Fractions of the T7-DNA were calculated based on the band intensity of T7- and random-DNA observed on the gel.

travels down the mRNA strand from the start codon to the end of the open reading frame (ORF) and pauses immediately before the Pu-linker/mRNA duplex region. Finally, the attached Pu attacks the nascent polypeptide to form a peptide–Pu-linker/mRNA complex. It should be noted that the UAG codon, which is assigned as a blank due to the lack of RF1, is placed to enhance the stalling of the ribosome immediately before the Pu-linker/mRNA duplex region (*vide infra*).

Stability of the Peptide–Pu-Linker/mRNA Complex. One of the initial concerns around TRAP display was the stability of the peptide–Pu-linker/mRNA complex because the Pu-linker is noncovalently attached to the mRNA. This noncovalent linkage may cause an exchange of polypeptide–Pu-linkers between different mRNAs, resulting in disarrangement of the one-to-one correspondence between the mRNA and encoded polypeptide. To evaluate the stability of the peptide–Pu-linker/mRNA complex throughout the TRAP display selection processes, we performed a T7-peptide pull-down assay, as shown in Figure 2a. Pu-linker/mRNA complexes of the T7-peptide-encoding mRNA (T7-mRNA) and random peptide-encoding mRNAs (random-mRNAs), both of which have the same Pu-linker annealing sequence at their 3′-UTR, were mixed in a 1:10 ratio, and after translation and RT using this template mRNA mixture, a T7-peptide–Pu-linker/mRNA/cDNA complex was selectively recovered using anti-T7 antibody-immobilized beads. Ideally, the T7-peptide–Pu-linker is attached only to the T7-mRNA and only the desired T7-mRNA/cDNA is recovered; but if the formed T7-peptide–Pu-linker is dissociated from the T7-mRNA and is reattached to random-mRNAs, a significant amount of the undesired random-mRNA/cDNAs would also be recovered (Figure S1).

Before the pull-down assay, we first tested whether the T7-DNA (Figure 2b, lane 1, 94 mers) and the random-DNA (Figure 2b, lane 2, 103 mers) are synthesized from the corresponding mRNAs by reverse transcription–polymerase chain reaction (RT-PCR) with similar efficiency. Indeed, the ratio of band intensity between the amplified T7-DNA and the amplified random-DNAs was proportional to that of the amount of their respective input DNA templates (Figure 2b, lanes 3 and 4, lower bands vs upper bands).

We then examined the stability of the peptide–Pu-linker/mRNA complexes of the conventional ligation-based mRNA display (Figure 1c, Li13) and those of ligation-free 13-mer annealing-based (Figure 1c, An13). In this experiment, we first used an orthogonal Phe-$tRNA_{CUA}$ prepared by a flexible tRNA acylation ribozyme (flexizyme)[10,22,23] to prepare a UAG codon code for Phe in the translation reaction. As expected, when the Li13-type Pu-linker/mRNA complexes were used, the T7-mRNA/cDNA was recovered in a highly selective manner using the anti-T7 antibody-immobilized beads (Figure 2b, lanes 4 vs 5). However, when the An13-type Pu-linker/mRNA complexes were used, a significant amount of random-mRNA/cDNAs was also recovered together with the T7-mRNA/cDNA (Figure 2b, lane 6). We considered that this result was presumably because of insufficient stability of the duplex formed between the Pu-linker DNA and the An13-type mRNA. To further stabilize the duplex, we increased the number of base pairs in the Pu-linker DNA/mRNA duplex from 13 mers to 21 mers and prepared the An21-type Pu-linker/mRNA, as shown in Figure 1b. However, this improvement only increased the fraction of the recovered T7-mRNA/cDNA from 34% to 40% (Figure 2b, lanes 6 vs 7). This indicated that more than half of the T7-peptide–Pu-linker dissociated from the T7-mRNA either during translation, RT, or the T7-peptide pull-down process. Given that the Pu-linker DNA/mRNA duplex was stable during RT and the pull-down process, and under even more stringent conditions (Figures S2 and S3), the dissociation must have occurred during translation. We hypothesized that the helicase activity of the translating ribosome caused the dissociation. To reduce the effect of the ribosome helicase activity by stalling the ribosome before the Pu-linker DNA/mRNA duplex region, we placed a UAG blank codon immediately before the duplex region in the mRNA by omitting the Phe-$tRNA^{Asn\text{-}E2}_{CUA}$ from the translation mixture (Figure 1b). As a result, the fraction of the recovered T7-mRNA/cDNA was significantly increased from 34% to 63% in the An13-type complex and from 40% to 73% in the An21-type complex (Figure 2b, lanes 6 vs 9 and lanes 7 vs 10, respectively). This indicated that the major cause of the T7-peptide–Pu-linker dissociation from the T7-mRNA was indeed ribosome helicase activity, and extending the Pu-linker DNA/mRNA duplex length and placing a blank UAG

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codon immediately before the duplex sufficiently stabilized the T7-peptide–Pu-linker/mRNA complexes.

Efficiency of the Random-Peptide–Pu-Linker/mRNA Complex Formation in the TRAP System. For selection of peptides from a random library, the efficiency of the random-peptide–Pu-linker/mRNA complex formation is highly important because it directly determines the diversity of the peptide library. To evaluate the efficiency of the complex formation, we added biotin-Phe (Figure 3a) charged onto

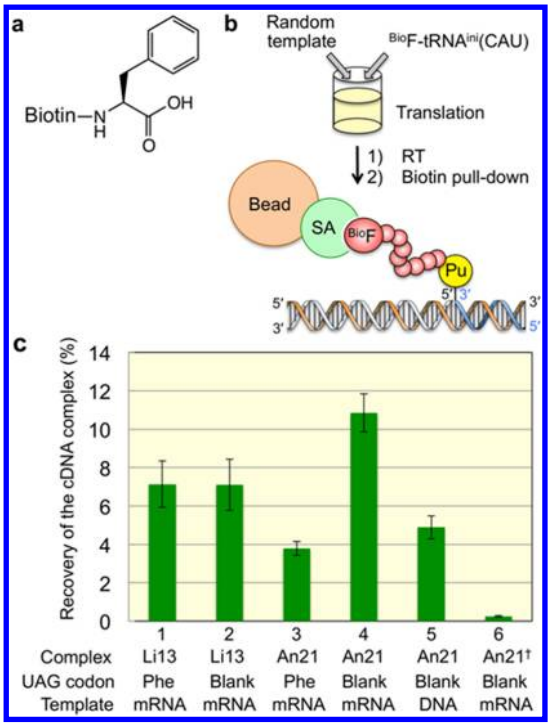

Figure 3. Analysis of the efficiency of the random-peptide–Pu-linker/mRNA complex formation in the TRAP system. (a) Structure of the biotin-L-Phe (^{Bio}F) used to synthesize biotin-label peptides. (b) Scheme of the biotin pull-down assay. The N terminus of synthesized peptides were labeled with biotin using ^{Bio}F-tRNA^{fMet}(CAU). After translation and reverse transcription, the biotin-peptide–Pu-linker/mRNA/cDNA complexes were captured by streptavidin magnetic beads. The recovered complexes were quantified by real-time PCR. (c) Efficiency of the random-peptide/mRNA complex formation. The reaction was performed in the translation mixture where a UAG codon is reassigned to Phe (columns 1 and 3) or a blank (columns 2, 4–6). The following templates were used: columns 1 and 2, Li13-type mRNAs; columns 3, 4, and 6, An21-type mRNAs; column 5, An21-type DNA templates. Dagger (†) indicates no incubation. Recovery of cDNA complexes was calculated by dividing the amount of recovered cDNA by the theoretical amount of mRNA/Pu-linker (1 μM) in the reaction mixture. Error bars represent standard deviation calculated from experiments performed in triplicate.

tRNA$^{fMet}_{CAU}$ by flexizyme to a translation mixture from which Met had been removed to label synthesized peptides with biotin at the N terminus (Figure 3b). Consequently, only the mRNAs that display biotin-peptides can be selectively recovered by pull-down using SA beads.

To compare the efficiency of random-peptide–Pu-linker/mRNA complex formation between TRAP display and conventional mRNA display, we first added the Li13- or An21-type Pu-linker/mRNA complex as a template to the translation mixture and performed the biotin pull-down experiment. Quantification of recovered cDNA by real-time PCR revealed that 7% of the Li13-type mRNA was recovered when a UAG codon was assigned to Phe and when it was assigned as a blank (Figure 3c, columns 1 and 2). However, recovery of the An21-type mRNA markedly decreased from 11% to 4% by reassignment of the UAG codon from blank to Phe (Figure 3c, columns 3 and 4). This suggested that the ribosome could dissociate the Pu-linker DNA/mRNA duplex in the An21-type Pu-linker/mRNA complex before Pu attacked the nascent peptide, but placing the blank codon immediately before the duplex region promoted the Pu attacking the nascent peptide by stalling the ribosome at the blank codon. Lower recovery of the Li13-type mRNA compared with the An21-type mRNA (Figure 3c, columns 2 and 4) could result from the moderate amount of appropriately ligated Li13-type Pu-linker/mRNA (data not shown).

We then evaluated the efficiency of the An21-type peptide–Pu-linker/mRNA complex formation by using the corresponding DNA instead of mRNA as a template in the TRAP system. To omit the time required to purify PCR-amplified template DNAs in every selection round, we first optimized the TRAP system for coupled transcription–translation. PCR mixture was directly added to the translation mixture without purification, and the transcribed mRNA was quantified by RT real-time PCR. We confirmed that a sufficient amount of mRNA (<1 pmol/μL) was produced after incubation for 5–10 min in the TRAP system containing 1 μM T7 RNA polymerase (Figure S4). To this TRAP system, we added the An21-type random-DNA as a template together with biotin-Phe-tRNA$^{fMet}_{CAU}$ and performed the biotin pull-down experiment. Recovery of the An21-type peptide/mRNA complex generated from the DNA template was reduced by half compared with that obtained from the mRNA template because the ribosomes may be consumed by the overproduced Pu-linker-free mRNA; however, this was still comparable with that of the Li13-type mRNA (Figure 3c, columns 2, 4, and 5). This level of efficiency would particularly be acceptable for the second and later rounds of selection, at least to ensure diversity in a polypeptide library, because active sequences are enriched and amplified in the previous rounds of selection. Therefore, we decided to use the An21-type random-mRNA as a template for the first round of selection to ensure library diversity and the amplified DNA as a template for the later rounds to accelerate the speed of selection.

Selective Enrichment for the T7-Peptide–Pu-Linker/mRNA Complex. To evaluate the ability of TRAP display to selectively enrich desired peptides, we compared the enrichment in TRAP display (An21-type) and conventional mRNA display (Li13-type) using a T7-peptide pull-down assay as described above. The Pu-linker/mRNA complexes of the T7-mRNA and random-mRNAs were mixed in a 1:3000 ratio and were added to the TRAP system. After the pull-down assay, RT-PCR products were analyzed using native polyacrylamide gel electrophoresis (PAGE).

Quantitation of the ratio of T7-DNA and random-DNA in the gel showed that the T7-mRNA was enriched by 3,700-fold in the conventional mRNA display selection (Figure 4, lanes 4 and 5) and by 4,900-fold in the TRAP display selection (Figure 4, lanes 6 and 7). This result revealed that TRAP display using the mRNA templates could selectively enrich the T7-DNA with similar or even improved efficiency over that of conventional mRNA display.

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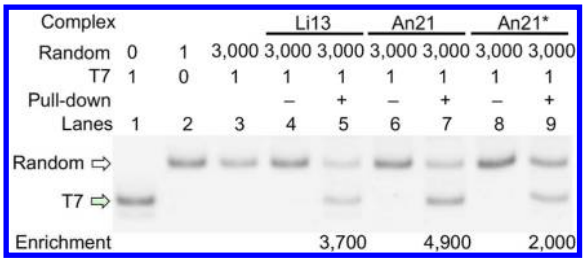

Figure 4. Comparison of enrichment of the T7-peptide template between conventional mRNA display (Li13) and TRAP display (An21). Lanes 1–3 contain DNA markers synthesized from T7-mRNA, random-mRNA, and a 1:3000 mixture of T7- and random-mRNAs. The T7- and random-mRNAs (or DNA) were mixed in a 1:3000 ratio in the translation system, where a UAG codon is reassigned to Phe (lanes 4, 5) or blank (lanes 6–9). The following templates were used in translation: lanes 4 and 5, Li13-type mRNAs; lanes 6 and 7, An21-type mRNAs; lanes 8 and 9, An21-type DNAs. Enrichment of the T7-peptide template was calculated from the band intensity observed on the gel before and after pull-down.

We also tested DNA as a template for selective enrichment of the T7-peptide–Pu-linker/mRNA complex. The T7-DNA and random-DNAs were added to the TRAP system in a 1:3,000 ratio. The result of the T7-peptide pull-down assay revealed that the T7-DNA was enriched by 2,000-fold (Figure 4, lanes 8 and 9), which indicated that TRAP display could generate the polypeptide-Pu-linker/mRNA from not only mRNA templates but also DNA templates and could enrich a target binding polypeptide by pull-down experiments using target-immobilized beads.

***In Vitro* Selection of Macrocyclic Peptides.** To demonstrate the ability of TRAP display to select functional peptides, we selected macrocyclic peptides against HSA. We used a macrocyclic peptide library[24] because the constrained macrocyclic structure often afforded high-affinity to targets.[25−30] For the preparation of a macrocyclic peptide library, we synthesized a novel amino acid for macrocyclization, *N*-[3-(2-chloroacetamido)benzoyl]-L-phenylalanine (ClAB-L-Phe) (Figure 5a), and we reassigned the AUG start codon to ClAB-L-Phe by adding ClAB-L-Phe-tRNA$^{fMet}_{CAU}$ to a Met-depleted TRAP system. We also prepared an mRNA library for peptides consisting of 8–12 random amino acids flanked by ClAB-L-Phe and Cys. The encoded peptides are spontaneously macrocyclized via a thioether linkage formed between the chloroacetyl and thiol groups[31] (Figure 5b).

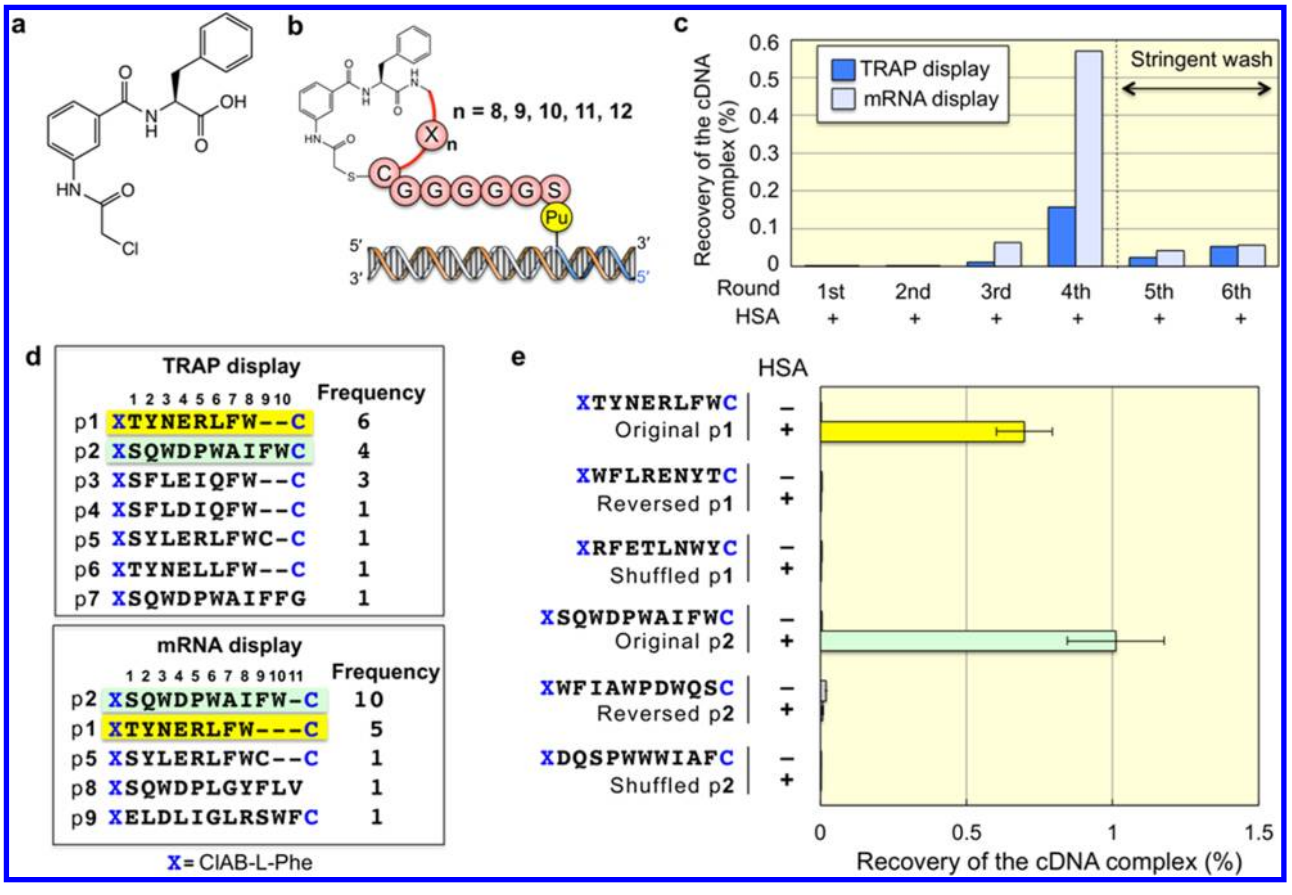

Figure 5. *In vitro* selection of macrocyclic peptides using TRAP display and mRNA display. (a) Structure of *N*-[3-(2-chloroacetamido)benzoyl]-L-Phe (ClAB-L-Phe) used for peptide macrocyclization. (b) Macrocyclic peptide library used in this study. Random mRNA/Pu-linker complexes displaying the encoded macrocyclic peptides consisting of 8–12 random amino acids flanked by ClAB-L-Phe and Cys. The chloroacetyl group of ClAB-L-Phe and the thiol group of Cys spontaneously form an intramolecular thioether bond. (c) Progress of TRAP display selection and mRNA display selection using HSA-immobilized SA beads. Recovery of the cDNA complexes was calculated in the same way as in Figure 3. (d) Sequences of selected peptides. The X and C highlighted in blue indicate ClAB-L-Phe and Cys, respectively. (e) Evaluation of selected peptides. Selected peptides (p**1** and p**2**) and the derivative peptides with reversed and shuffled sequences of the original peptides were prepared as the An21-type complex. The resulting complexes were examined by a pull-down assay using HSA-immobilized or HSA-free SA beads.

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The library of macrocyclic peptides displayed on the An21-type Pu-linker/mRNA complexes was generated in the translation mixture and used in the pull-down against HSA immobilized on SA beads. The recovered DNA products, amplified by RT-PCR, were directly added to the TRAP system without purification, and the resulting macrocyclic peptide library was used for the next round of selection. Since cDNA recovery was significantly increased at the fourth round of selection (Figure 5c), we performed a stringent wash in the following two rounds to enrich peptide binders with higher affinity. Notably, the six rounds of selection were performed in approximately 14 h, which proved that TRAP display greatly increased the speed of peptide selection.

To establish whether the conventional mRNA display selection produces the same macrocyclic peptides obtained by the TRAP display selection, we also performed mRNA display selection of macrocyclic peptides (Figure 5c). The sequence alignment of selected peptides obtained by mRNA display and TRAP display showed that the two most abundant sequences in each display method were the same (Figure 5d, p**1** XTYNERLFWC and p**2** XSQWDPWAIFWC, where X indicates ClAB-L-Phe), which confirms the reliability of TRAP display. The results of pull-down using HSA-immobilized or HSA-free SA beads followed by quantification of the recovered cDNA by real-time PCR showed that the p**1** and p**2** macrocyclic peptides indeed bound to HSA-immobilized SA beads but not to the free SA beads (Figure 5e). Moreover, macrocyclic peptides consisting of reversed or shuffled sequences of p**1** or p**2** did not bind to the HSA-immobilized SA beads (Figure 5e). These results indicated that not only the composition but also the sequence of the amino acids in p**1** and p**2** were important for binding to HSA-immobilized SA beads. We also chemically synthesized the fluorescent-labeled p**1** and p**2** by Fmoc solid-phase synthesis and determined their affinity to the target by using fluorescence polarization assay. Both peptides bound to HSA−SA complex with nanomolar affinity [K_d (App) = 41 and 43 nM for p**1** and p**2**, respectively] (Figure S5, see Supplementary Note). Most importantly, these results show that TRAP display can produce macrocyclic peptide leads with nanomolar affinity to their target in as little as 14 h.

DISCUSSION

In this study, we developed a novel *in vitro* selection technology that greatly accelerated the generation of functional polypeptides with desired properties. First, we constructed the TRAP system in which the steps of transcription, association of a Pu-linker, translation, and peptidyl-transfer reaction of polypeptide to the Pu are sequentially performed in a single reaction mixture (Figure 1a). Because the Pu-linker/mRNA complex is maintained via a noncovalent linkage, the complex may not be sufficiently stable to be used for selection, and thus we evaluated the stability of the polypeptide-Pu-linker/mRNA complexes. Although the complex was stable under various conditions, such as RT and pull-down, it became dissociated in the translation mixture. Based on the hypothesis that the ribosome helicase activity causes dissociation of the Pu-linker from the mRNA, we placed a blank UAG codon immediately before the Pu-linker DNA/mRNA duplex region and successfully prevented complex dissociation (Figure 2b, lanes 7 and 10). Moreover, the placement of this UAG blank codon also increased the efficiency of the An21-type peptide/mRNA complex formation from 4% to 11% (Figure 3c, columns 3 and 4), whereas no efficiency change was observed for the Li13-type complex (Figure 3c, columns 1 and 2). The blank codon, which stalls the ribosome, therefore, plays two key roles in TRAP display selection: (1) stabilizing the Pu-linker/mRNA complex to prevent dissociation of the peptide-Pu-linker from a particular mRNA and subsequent reassociation with a different mRNA, and (2) increasing the efficiency of the peptide/Pu-linker/mRNA complex formation by providing a delay for Pu attacking the nascent peptide (Figure S6).

The biotin pull-down experiment also provided insight into the diversity of the polypeptide library. To our knowledge, no investigations on *in vitro* display technologies have evaluated the display efficiency of random peptides. Our attempt to evaluate the inherent display efficiency of random peptides revealed that 11% of the random pool of mRNA was converted into a peptide−Pu-linker/mRNA complex (Figure 3c, column 4). This discovery suggests that highly diverse polypeptide libraries (6×10^{13} molecules/mL) can be produced using TRAP display selection.

To reveal the reliability of TRAP display, we compared the results of selective enrichment and *in vitro* selection of TRAP display with those of conventional mRNA display. We observed better enrichment in mRNA-based TRAP display than in conventional mRNA display (Figure 4, lanes 5 and 7), and the enrichment reduced only 2-fold when a DNA template was used (Figure 4, lanes 7 and 9). These results are in agreement with the efficiency of the peptide−Pu-linker/mRNA complex formation where 70% and 50% of relative efficiency were observed in the Li13-type mRNA and the An21-type DNA, respectively, as compared with the An21-type mRNA (Figure 3c, columns 1 and 5 vs 4). The relatively lower enrichment of TRAP display using DNA templates was also observed in macrocyclic peptide selection (Figure 5c). However, this difference in enrichment between mRNA display and TRAP display did not affect the results of the selection (Figure 5d).

Fluorescence polarization assay using chemically synthesized peptides revealed that the selected peptides bound to the HSA−SA complex with nanomolar affinity. In future studies, the use of alternative types of target immobilization methods, such as amine coupling or His-tag mediated immobilization, should be investigated. Selection of more potent HSA binders and the application of such peptides for therapeutic use[32−36] would be of significant interest.

CONCLUSIONS

We have developed the TRAP system in which stable polypeptide-mRNA complexes are automatically formed by addition of the DNA template. In the TRAP system, transcription−translation is coupled with association of a Pu-linker and conjugation of the nascent polypeptide and puromycin. A blank UAG codon placed immediately before the Pu-linker DNA/mRNA duplex region to stall the ribosome prevents dissociation of the Pu-linker DNA/mRNA complex and increases the efficiency of the peptide/mRNA complex formation. Using TRAP display, we completed six rounds of selection in approximately 14 h and successfully obtained high affinity macrocyclic peptides that bind to HSA−SA complex. The ease of TRAP display should make it amenable to automation, in which 10s to 100s of libraries could be run in parallel, something that is not possible to do with conventional mRNA display.

As TRAP display greatly accelerates the speed of functional polypeptide selection, it will facilitate the discovery of the next

generation of peptides, peptidomimetics, and proteins that could be useful for biological and therapeutic applications in the future.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures and additional tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Authors Patrick C. Reid and Hiroshi Murakami declare competing financial interests, as both are founders and shareholders in PeptiDream Inc, a peptide drug discovery company using similar technology.

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