Published online 15 June 2009

Nucleic Acids Research, 2009, Vol. 37, No. 16 ***e108***
doi:10.1093/nar/gkp514

cDNA display: a novel screening method for functional disulfide-rich peptides by solid-phase synthesis and stabilization of mRNA–protein fusions

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Received January 4, 2009; Revised April 27, 2009; Accepted May 29, 2009

ABSTRACT

We report a robust display technology for the screening of disulfide-rich peptides, based on cDNA–protein fusions, by developing a novel and versatile puromycin-linker DNA. This linker comprises four major portions: a 'ligation site' for T4 RNA ligase, a 'biotin site' for solid-phase handling, a 'reverse transcription primer site' for the efficient and rapid conversion from an unstable mRNA–protein fusion (mRNA display) to a stable mRNA/cDNA–protein fusion (cDNA display) whose cDNA is covalently linked to its encoded protein and a 'restriction enzyme site' for the release of a complex from the solid support. This enables not only stabilizing mRNA–protein fusions but also promoting both protein folding and disulfide shuffling reactions. We evaluated the performance of cDNA display in different model systems and demonstrated an enrichment efficiency of 20-fold per selection round. Selection of a 32-residue random library against interleukin-6 receptor generated novel peptides containing multiple disulfide bonds with a unique linkage for its function. The peptides were found to bind with the target in the low nanomolar range. These results show the suitability of our method for *in vitro* selections of disulfide-rich proteins and other potential applications.

INTRODUCTION

Phage display techniques are powerful tools for selecting peptides and proteins that bind with high affinity and specificity to target molecules from random sequence libraries (1). Recently, several *in vitro* display techniques (genotype–phenotype linkage technology) have been developed using cell-free translation systems to overcome the difficulties associated with conventional cellular and phage-based display methods. The main potential advantage of using *in vitro* methods is to generate large libraries and thus increase the diversity of the proteins being screened by four or more orders of magnitude compared with phage-display methods (2).

In recent years, both ribosome display (3,4) and '*in vitro* virus' mRNA display (5,6) have already been proved to be very successful and practical *in vitro* techniques for exploring functional peptides and proteins from large libraries (7). These methods have enabled the binding of mRNA molecules to their encoded protein products via a ribosome or a puromycin molecule. Owing to the simple formation of mRNA–protein fusions in this instance, these molecules can be applied not only in evolutionary

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molecular engineering studies, but also in genome-wide proteomics and analyses of protein function (8,9). However, mRNA-dependent technology has so far been perceived as technically challenging owing to the labile nature of RNA molecules. For example, the application of mRNA display techniques for the analysis of cell surface antigens can be presumably problematic due to ribonuclease activity of the cells. On the other hand, phage display has proved to be very robust and successful in the screening of antibody fragments directed against cell-surface proteins that are important drug targets, such as G protein-coupled receptors (GPCR) (10,11). To increase the stability of the mRNA display products (i.e. mRNA–protein fusion molecules), the labile aspect of the mRNA region has been improved by the additional synthesis of its cDNA counterpart and the generation of an mRNA/cDNA–protein fusion molecule using a puromycin–linker DNA harboring a primer for reverse transcription (RT) (12,13). Although these methods facilitate ready RT without the addition of primers into the lysate, it is still necessary to rapidly purify the mRNA–protein fusion molecules to avoid any further degradation by nucleases that may have contaminated the cell-free translation reaction and also to release the ribosome that remains bound to the coding regions of the mRNAs.

In this article, we report a novel screening method by using a puromycin-linker DNA containing a 'ligation site', a 'biotin site' 'reverse transcription primer site' and a 'restriction enzyme site' described in the previous report (14). These features facilitate extremely rapid ligation of mRNA and linker, biotin/streptavidin-based purification and cDNA synthesis by RT that together prevent degradation of mRNA, significantly reduce the execution time, allow conversion of mRNA–protein fusion (mRNA display) to cDNA–protein fusion (cDNA display), where the cDNA remains covalently linked to their encoded protein and facilitates solid-phase handling (Figure 1). Thus, disulfide-rich proteins can also be expressed and refolded by easy and rapid buffer exchange. The cDNA display enables *in vitro* selection of various kinds of peptide and protein libraries of more than 10^{12} different sequences comparable to or higher than other *in vitro* display techniques.

We validated the performance of this novel puromycin–linker DNA in a screening for test proteins (immunoglobulin G, POU-specific DNA-binding domain of Oct-1 and anti-FLAG antibody). Selection against interleukin-6 receptor (IL-6R) using a 32-residue random peptide library allowed us to identify novel peptides with multiple disulfide bonds that have not been reported by other methods. We also found and showed that one of the selected peptides requires a unique disulfide linkage for its function.

MATERIALS AND METHODS

Chemicals and reagents

The modified oligonucleotides 'Puro-F-S' and 'Biotin-loop' were obtained from Geneworld (Tokyo). 'Puro-F-S' stands for 5′-(S)-TC(F)-(Spec18)-(Spec18)-(Spec18)-(Spec18)-CC-(Puro)-3′, where (S): 5′-Thiol-ModifierC6, (F): Fluorescein-dT, (Puro): Puromycin CPG and (Spacer18): Spacer Phosphoramidite 18. 'Biotin-loop' stands for 5′-CCCGGTGCAGCTGTTTCATC(T-B)CGGAAACAGCTGCACCCCCCGCCGCCCCCCG(T)CCT-3′, where (T): Amino-Modifier C6 dT, and (T-B): Biotin-dT (underline indicates a recognition sequence for the PvuII restriction enzyme). EMCS [N-(6-Maleimidocaproyloxy) succinimide] was purchased from Dojindo (Kumamoto, Japan). TCEP [Tris (2-Carboxyethyl) phosphine] was purchased from Pierce.

Vectors, oligonucleotides and library construction

The B domain of protein A (15) was obtained from the pEZZ 18 protein A gene fusion vector (GE Healthcare). The primers used were as follows: a forward primer incorporating the T7 promoter, the 5′ untranslated region 'omega' of tobacco mosaic virus (TMV), a Kozak sequence and an ATG start codon; a reverse primer harboring a hexa-histidine tag, a spacer sequence (GGGGGAGGCAGC) and a complementary sequence (AGGACGGGGGGCGGGGAAA) for the puromycin–linker DNA at the 3′-terminus to enable ligation between the mRNA and the puromycin–linker DNA. In the case of the Pou-specific DNA-binding domain of Oct-1 (PDO) (16), the template was generated by replacing the B domain region with PDO.

For the affinity selection of the anti-FLAG M2 antibody, two single-stranded synthetic DNAs (27-mers) encoding the FLAG epitope and a random decamer peptide were purchased from the FASMAC Corporation (Japan). The DNA encoding the random decamer peptide comprised the codon triplets XYZ, where X, Y and Z indicate nucleotide mixtures, and the mixing ratios were X: T 13%, C 20%, A 35%, G 32%; Y: T 24%, C 22%, A 30%, G 24%; and Z: T 37%, C 37%, A 0%, G 26% (17). Similarly, the 35 residues library was prepared by DNAs encoding the 32 random residues using codon triplets as mentioned above.

Synthesis of puromycin–linker DNA

The modified oligonucleotides: 'Puro-F-S' and 'Biotin-loop' were obtained from Geneworld (Tokyo) and BEX (Tokyo). 'Puro-F-S' stands for 5′-(S)-TC(F)-(Spec18)-(Spec18)-(Spec18)-(Spec18)-CC-(Puro)-3′, where (S):5′-Thiol-Modifier C6, (F): Fluorescein-dT, (Puro): Puromycin CPG and (Spacer18): Spacer Phosphoramidite 18. 'Biotin-loop' stands for 5′-CCCGGTGCAGCTGTTTCATC(T-B)CGGAAACAGCTGCACCCCCCGCCGCCCCCCG(T)CCT-3′, where (T): Amino-Modifier C6 dT and (T-B): Biotin-dT (underline indicates a recognition sequence for the PvuII restriction enzyme).

The 5′-thiol group of 'Puro-F-S' (10 nmol) was reduced by 1mM TCEP in 100 µl of 50 mM phosphate buffer (pH 7.0) for 6 h at room temperature and then desalted on a NAP-5 column (GE Healthcare) just before use. Total 10 nmol of 'Biotin-loop' and 2 µmol EMCS were then added to 100 µl of 0.2 M sodium phosphate buffer, pH 7.0. The mixture was subsequently incubated for 30 min at 37°C and ethanol precipitation was performed at 4°C to remove excess EMCS. The precipitate was washed twice

with 500 µl of pre-cooled 70% ethanol and dissolved in 10 µl of pre-cooled 0.2 M sodium phosphate buffer (pH 7.0). The reduced 'Puro-F-S' was immediately added and stirred at 4°C overnight. The reaction was stopped by adding 4 mM TCEP for 15 min at 37°C. Ethanol precipitation was then performed to remove the excess 'Puro-F-S' at room temperature. To remove the 'Biotin-loop' and the uncrosslinked Biotin-loop–EMCS complexes, the precipitate was dissolved with 0.1 M TEAA (Glen Research) or phosphate buffer and purified with a C18 HPLC column under the following conditions: column: AR-300, 4.6 × 250 mm (Nacalai Tesque, Japan); solvent A: 0.1 M TEAA; solvent B: acetonitrile/water (80:20, v/v); gradient; B/A(15–35%, 33 min); flow rate: 0.5 ml/min; detection by absorbance at 254 nm and 490 nm. The fraction from the last peak at an absorbance of 254 nm (corresponding to a single peak at an absorbance of 490 nm) was collected. After drying, the 'puromycin–linker DNA' was resuspended in diethylpyrocarbonate (DEPC)-treated water and stored.

Immobilization of mRNA–puromycin conjugates on magnetic beads

Streptavidin-coated magnetic beads of 2.3 µm in size (MAGNOTEX-SA, Takara) were washed twice with binding buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 M NaCl, 0.1% Triton X-100) according to the manufacturer's instructions. The mRNA–puromycin conjugate (48 pmol) and SA beads (1.2 mg) were then incubated in 120 µl of binding buffer for 10 min at room temperature. The beads were subsequently washed once each with binding buffer and translation mix buffer (Ambion), prior to cell-free translation.

Cell-free translation and RT on magnetic beads

After separation of the magnetic beads with a magnetic stand, 300 µl of cell-free translation extracts (Ambion) were added and the mixture was incubated at 30°C for 20 min. To increase the yield of mRNA–protein fusions, the post-translation fusion reaction was performed for an additional incubation of translated product at 37°C for 90 min in the presence of high-salt concentrations (KCl and $MgCl_2$ to final concentrations of 750 and 63 mM, respectively). The beads bounded with the expressed mRNA–protein complex were then washed twice with 200 µl of binding buffer containing RNase inhibitor (SUPERaseIn, Ambion) and rinsed with 100 µl of RT buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$). RT was performed at 42°C for 10 min by adding 80 µl of RT buffer and 80 units of reverse transcriptase M-MLV (Takara) to the beads.

Refolding of disulfide-rich proteins

The cDNA–protein fusions on streptavidin-coated magnetic beads were reduced by the addition of 100 mM DTT for 1 h at 25°C and washed with binding buffer (Takara). Refolding was performed in the presence of 1 mM oxidized glutathione, 10 mM reduced glutathione and protein disulfide isomerase at an equimolar ratio with the input cDNA fusions for 1 h at 25°C. The beads were washed and subjected to PvuII digestion (mentioned below) and Ni–NTA purification followed by selection.

Collection of cDNA–protein fusion molecules from magnetic beads

The magnetic beads bounded with the reverse transcribed mRNA–protein fusions were washed once with binding buffer followed by 'M buffer' (10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl; TaKaRa) and separated using a magnetic stand. The release of the mRNA/cDNA–protein fusion molecules from the beads was performed at 37°C for 1 h by the addition of M buffer (40 µl) and 24 units of PvuII (Takara) and BSA (final concentration 0.1 mg/ml). BSA was added to reduce any non-specific interactions between the mRNA/cDNA–protein fusion products and the beads. The cDNA–protein fusion products were then purified from the remaining supernatant using Ni–NTA agarose beads (QIAGEN).

For detection, after separation of the beads from this reaction, the supernatant was transferred to a new tube and mRNA was removed by incubation with 2 units of ribonuclease H (Promega) at 37°C for 20 min and subjected to SDS–PAGE containing 6 M urea. Fluoroimager (Typhoon 8600) was then used to view and quantify the bands on the gel by the detection of the FITC label attached to the puromycin–linker.

Protein immobilization and labeling

Immunoglobulin G (IgG; Sigma), IL-6R (Peprotech) and bovine serum albumin (BSA; Ambion) (50 µg) were coupled to 200 µl of NHS-activated Sepharose 4 Fast Flow (GE Healthcare) according to manufacturer's instructions. The final solution was prepared as 50% slurry. Non-coated beads were also prepared by the same method in the absence of protein.

The double-stranded DNA harboring the Pou binding site was prepared by the hybridization of the oligonucleotides 5′-CCAGGGTATGCAAATTATTAAGGGCAAAAA-biotin-3′ and 5′-TTTTTGCCCTTAATAATTTGCATACCCTGG-3′. The underlined sequences indicate the Pou binding site (16). The resulting biotinylated dsDNA (hereby referred to as Oct-DNA) was then immobilized on streptavidin-coated sepharose beads (GE Healthcare). Biotin labeling of IL-6R was performed using NHS–ss–biotin (Pierce) according to manufacturer's instructions. The resultant mixture was dialyzed extensively against buffer to remove the free biotin molecules. Protein concentration was estimated by SDS–PAGE using known concentration of IL-6R.

In vitro affinity selection

Three different templates for cDNA display, the B domain of protein A (BDA), Pou-specific DNA binding domain of Oct-1 (PDO) were prepared for test screenings by the use of human immunoglobulin G (IgG) and dsDNA containing the Pou binding site (Oct-DNA), respectively.

A cDNA library containing BDA/PDO (1:1, 1:3 and 1:20) was prepared by translation of the linker-conjugated

mRNAs in the mentioned ratios, incubation with high salt, RT, PvuII digestion and Ni–NTA purification via the 6× His tag. The purified fractions of BDA/PDO libraries were added to either IgG-coated beads or to Oct-DNA-coated beads (20 µl of 50% slurry rinsed with selection buffer), respectively, in 100 µl of the selection buffer (50 mM Tris–HCl pH 7.6, 1 mM EDTA, 500 mM NaCl, 0.1% Tween 20) at room temperature for 30 min with rotation. The mixture was incubated at room temperature for 30 min with rotation. The beads were then recovered and washed several times with 500 µl of the selection buffer. The bound cDNA display molecules were removed from the beads with 100 µl of elution buffer [0.1M Glycine-HCl (pH 2.5)] and neutralized immediately with 1 M Tris–HCl (pH 8.0). The supernatant was precipitated with ethanol and coprecipitant (Quick-precip Plus, Edge BioSystems) and dissolved in 10 µl of water. An aliquot of 2 µl was amplified by PCR using 0.2 µM of the primers 5′-(FITC) –CAACAACATTACATTTTACATT CTACAACTACAAGCCACC-3′ and 5′-TTTCCCCGC CGCCCCCCGTCCTGCTTCCGCCGTGATGATGAT GATGATGGCTGCCTCCCCC-3′ for 25 cycles consisting of denaturation for 20 s, annealing for 15 s and elongation for 30 s and then quantitatively analyzed by denaturing gel electrophoresis (4.5% T and 8 M urea) and fluoroimager.

In order to validate the ratios between the selected PDO and/or BDA from a mixed pool against a given target, the selected DNAs were amplified for various cycles such as 15, 20, 25, 30 and 35 using the cycle conditions as mentioned above. We found that a linear range was achieved around 25 cycles and the linearity started to diminish around 30 cycles. Thus, we fixed the PCR cycles to 25.

In the case of FLAG-tag, affinity selection was performed using anti-FLAG M2 antibody-agarose beads (Sigma) for three rounds. PCR products of selected molecules from each round were analyzed by direct sequencing. The PCR products were also cloned into the TA vector (Qiagen) and clones were picked randomly for sequencing.

Selection of random library against IL-6R

A random library was prepared by overlap PCR of the fragments containing the randomized bases that encode 35 residues and essential components for the display of peptides such as 6×-His and Y-tag at the 3′-terminus and T7 promoter, 5′ UTR, Kozak and start codon at the 5′-terminus. Two hundred picomoles of initial library mRNA was linked to the puromycin linker and translated into 1 ml of lysate for 20 min at 30°C. Formation of protein fusions was achieved by the addition of 750 mM and 63 mM of KCl and $MgCl_2$, respectively. The successive steps (RT, *Pvu*II digestion and Ni–NTA purification) were scaled up using the pilot conditions as mentioned earlier. DTT was excluded from all the buffers used. The purified cDNA display peptides were estimated by the fluorescence intensity using known FITC-labeled oligonucleotides.

The first round of selection contained 2.7×10^{11} library molecules and 25 nM biotinylated IL-6R in S-buffer (50 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, 0.1% Tween, pH 7.6, 0.1 mg/ml BSA) and incubated for 1 h at room temperature. The mixture was then captured with 0.2 mg streptavidin (SA) beads and washed several times with S-buffer. The bound molecules were recovered by incubation with 100 mM DTT (Sigma) for 10 min, purified and processed to next round.

From round 4 (R4), random mutagenesis was introduced during the PCR amplification step of the selected molecules using the Diversify PCR Random Mutagenesis Kit (Clontech, CA, USA) designated as Scheme 1. Mutations were introduced at 3.2–7.3%. Selections were performed for nine rounds using these conditions (i.e. upto R12). In Scheme 2, after random mutagenesis, we performed solid-phase refolding of peptides in the presence of 1 mM oxidized glutathione (Sigma), 10 mM reduced glutathione (Sigma) and protein disulfide isomerase (PDI; Takara) added at 1:1 molar ratio with the protein for 1 h at room temperature. The beads were washed and the peptides were released from the beads by PvuII digestion and purified on Ni–NTA resin chromatography. Selections were performed for six rounds using these conditions (i.e. upto R9).

Peptide synthesis

Peptides containing two cysteine residues (Cys-2 type) composed of one disulfide bond and those containing four cysteine residues (Cys-4 type) composed of two disulfide bonds between C1 and C4, and C2 and C3 were prepared by the chemical synthesis. Additionally, Cys4-2B that was composed of a different disulfide pattern (i.e. C1 and C3, and C2 and C4) was also synthesized. In general, a biotin moiety was attached at the N-terminus. The Cys-2 peptides containing two Cys residues were custom synthesized by Toray (Tokyo, Japan) and the Cys4 peptides containing four Cys residues were synthesized and characterized by HPLC profiles for their topologies using retention time by Peptide Institute (Osaka, Japan) according to the previously reported method (18).

Binding assay

Binding assay of peptides. One µM of synthesized biotinylated peptides were incubated with 200 nM of immobilized IL-6R for 1 h at room temperature in phosphate buffered saline (PBS). The mixture was centrifuged and supernatant was discarded. The beads were washed thoroughly with PBS-T (Tween 20, 0.1%) and incubated with streptavidin–horseradish peroxidase (SA-HRP; GE Healthcare) at 1/2000 dilution for 30 min. After several washings with PBS-T, 200 µl of TMB substrate was added. The reaction was stopped with 0.5 M H_2SO_4 after proper color development. Absorbance was monitored at 450 nm.

Determination of dissociation constant (K_d). Binding affinity of the synthesized biotinylated peptides was assayed according to the previously reported method with a few modifications (19). A constant amount of peptide (10 nM) was incubated with varying amounts of IL-6R (1 nM to 1 µM) in PBS at 25°C for 1 h. The mixture was applied to a constant amount of IL-6R-coated beads (200 nM) and

incubated further for 30 min. After several washings with PBS-T, SA-HRP at 1/2000 dilution was added and incubated for 30 min. The supernatant was removed and the beads were washed four to five times with PBS-T. Two hundred microliter of the TMB substrate was added for color development and the reaction was stopped with 0.5 M H_2SO_4. Absorbance was monitored at 450 nm. Data were plotted using GraphPad Prism 4 (GraphPad software Inc., San Diego, CA, USA).

RESULTS

Characteristics of a puromycin–linker DNA construct, which facilitates the efficient synthesis of mRNA/cDNA–protein fusion products

The puromycin–linker DNA (Figure 1A) has four main characteristics that facilitate the efficient synthesis of stable mRNA/cDNA–protein fusion molecules. First, a 'ligation site' to prepare the mRNA–puromycin conjugates that consists of a complementary region that hybridizes with the 3′-terminal end of the mRNA template and a ligation site that promotes an efficient T4 RNA ligase reaction.

Second, it consists of a 'biotin site' for introducing solid-phase handling of the displayed products. Rapid purification of the mRNA–protein fusion products from the cell-free translation mixture is a prerequisite for the subsequent post-translation reactions and the screening steps. For this purpose, we incorporated a biotin site into the puromycin–linker DNA (Figure 1A).

Third, it consists of a 'RT primer site' for efficient and rapid conversion of a labile mRNA–protein fusion to a stable cDNA–protein fusion. With other display techniques, a DNA primer is added to the RT reaction that is heated to 80°C (or more) for several minutes in order to anneal to the mRNA and initiate the reaction. This process may denature the protein moiety of the mRNA–protein fusion molecule. We therefore included a primer region in our novel puromycin–linker DNA (Figure 1A) and omitted the heating step prior to the RT reaction. As a result, more than 90% of the cDNA was found to be synthesized within 5 min from the start of RT reaction without the initial heating step (Figure 2A). On the other hand, 60-min incubation was required to synthesize the cDNA in the conventional RT reaction using RT primer (5′-biotin-TTTCCCCGCCGCCCCCCGTC-3′) without the annealing step (Figure 2B).

Fourth, a 'restriction enzyme site' analysis for the release of the products after all the processing steps is performed. For this purpose, we incorporated a restriction enzyme site into the puromycin–linker DNA construct (Figure 1A). To confirm this, we examined the efficiency of digestion of the linker DNA construct immobilized on streptavidin-coated beads by the restriction enzyme PvuII and compared it with the digestion of a free linker (i.e. liquid phase). Equal amount of ligation product was reverse transcribed in the liquid-phase and solid-phase (i.e. onto the bead surface) modes, respectively. Following this, the product (mRNA/cDNA–puromycin–linker DNA) was treated by PvuII and the RNA region of product was degraded by RNase H successively for PAGE analysis (Figure 3A). As shown in Figure 3B, the efficiency of cleavage of fragment (ii) from fragment (i) in the liquid-phase mode is about 70% or more (lane 2). Nevertheless, for the solid-phase mode, where substrate is attached onto the surface, it is important to assess whether the effect of steric hindrance between the attached moiety (i.e. mRNA/cDNA/protein) and bead surface can influence the digestion reaction of PvuII, as the restriction site is close to the bead surface. The efficiency of RT and PvuII digestion to release RT product from the surface using the solid-phase reaction (lane 3) was comparable to that of the liquid-phase reaction (lower bands in lanes 2 and 3) and indicates that the beads do not cause any steric hindrance in the restriction cleavage of the DNA substrate.

Prior to the solid-phase performance of the puromycin–linker, we investigated the efficiency of the linker in the liquid phase. In general, 5–20% of the input mRNA template was observed to be converted to the mRNA–protein fusion product within the lysate (Figure 4A). The final yield was 0.1–0.3% after subjecting the templates to steps shown in Figure 1B (except that the templates were translated and fusion formation was in the liquid phase). The scheme of optimization and procedures to convert mRNA display to cDNA display by the solid-phase process is described in Figure 1B. After performing all the steps, we found that around 0.2% of the purified cDNA–protein fusion could be obtained based on the input mRNA–linker (Figure 4B).

Enrichment efficiency of affinity selection by cDNA display

To examine the performance of our new cDNA display method in the selection of a target molecule, we constructed two different DNA templates (Figure 5A). The B domain of protein A (BDA, 58 residues) (15) and the POU-specific DNA-binding domain of Oct-1 (PDO, 74 residues) (16) consist of three and four α-helical structures, respectively, and do not contain any disulfide bonds. The interaction between each pair, BDA/IgG and PDO/Oct-DNA is highly specific and could thus be specifically selected from a mixed pool.

The validity of our method was evaluated by designing a semiquantitative estimation of ratios between the selected PDO and BDA from a mixed pool against a given target (Figure 5B). Because puromycin-conjugated BDA and PDO interacts with IgG and Oct-DNA with dissociation constants of 13 nM (20) and 0.15 nM (21), we attempted to perform a selection against IgG from a pool comprising cDNA displayed PDO or BDA. Screening at an equimolar ratio of PDO and BDA (starting template concentrations) against Oct-DNA resulted in selection of ~20-fold more PDO molecules than BDA in a single round of selection. Similarly, screening against IgG yielded a 20-fold higher selected BDA compared to PDO [Figure 5C(a)]. In addition, screenings against IgG from pools of PDO and BDA in the ratio of 3:1 and 20:1, respectively, resulted in the selection of PDO and BDA in the ratios 1:7 and 1:1 that further confirms this selection efficiency

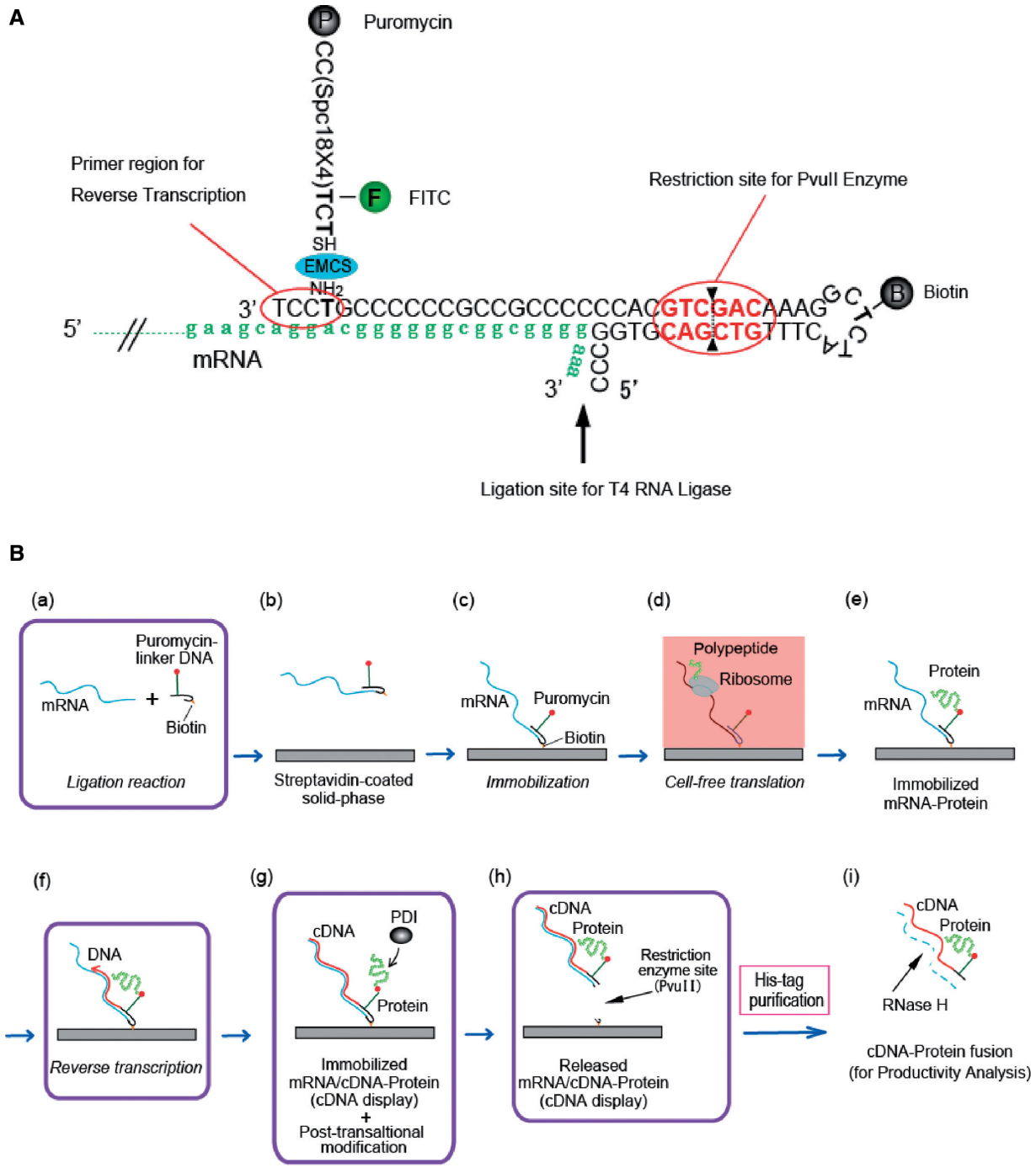

Figure 1. Schematic diagram of the puromycin–linker DNA construct and its utilities for selection. (**A**) The puromycin–linker DNA comprises four parts: a ligation site for mRNA, a primer region for RT, a biotin moiety for immobilization of mRNA–puromycin–linker conjugate to a solid surface using biotin–streptavidin chemistry and a restriction site for a release of the molecules from the solid surfaces. In addition, the linker has puromycin (for covalent linking of the expressed protein to mRNA) and FITC (for detection and quantification) moieties. (**B**) Schematic representation of the cDNA display method using the puromycin–linker DNA. After mRNA is ligated with the puromycin–linker DNA using T4 RNA ligase (a, b), the resulting product is immobilized on a streptavidin-coated surface (c). The immobilized mRNA is then translated in a cell-free system (d). The synthesized protein then fuses to its encoding gene via the puromycin moiety (e), and the immobilized mRNA is reverse transcribed using the primer region of the puromycin–linker DNA (f). A complete mRNA/cDNA–protein fusion product (cDNA display) is then displayed on the solid surface and proteins can be modified by post-translation reactions (g). The cDNA display molecule is released from its solid surface by cleavage with the PvuII enzyme (h) and can be purified via a C-terminal tag. To confirm the presence of cDNA–protein fusions, the mRNA is digested with RNase H (i). The outlined steps a, f, g, h are marked as key steps in order to convert mRNA display to cDNA display.

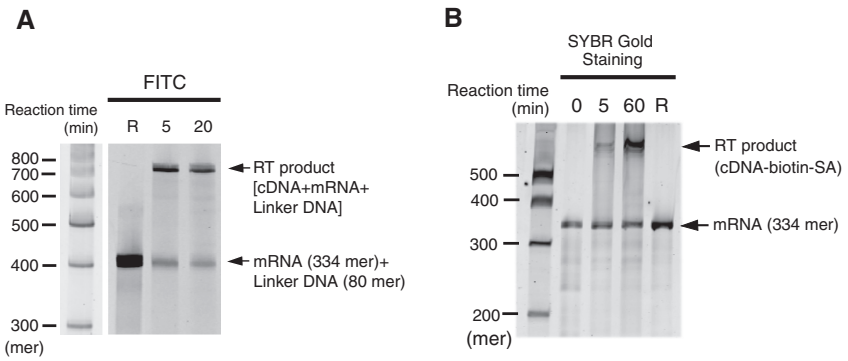

Figure 2. Characteristics of a novel puromycin–linker DNA: Reverse transcription (RT). Efficiency of RT was evaluated by comparison of the puromycin–linker DNA (**A**) versus a biotinylated primer (**B**). The BDA mRNA (334 bp) was ligated to the puromycin–linker and RT reactions were performed at 42°C for either 5 min or 20 min. After RT, the reverse transcribed single-stranded cDNA fused linker DNA–mRNA was analyzed by denaturing PAGE (6% T gel containing 8 M urea) and detected using FITC fluorescence (A). To compare these results with conventional RT (used in case of mRNA display), the same mRNA (334 b) (without the linker) was reverse transcribed after the addition of a biotinylated primer at room temperature for either 5 min or 60 min. All products were incubated at 70°C for 15 min to inactivate the reverse transcriptase. In order to resolve the reverse transcribed cDNA product from the mRNA template in conventional RT, the reaction was followed by incubation with streptavidin (SA) for 30 min as described (29) and the cDNA products attached to streptavidin (SA) were analyzed by denaturing PAGE (4% gel containing 8 M urea) and detected with SYBR Gold dye (B).

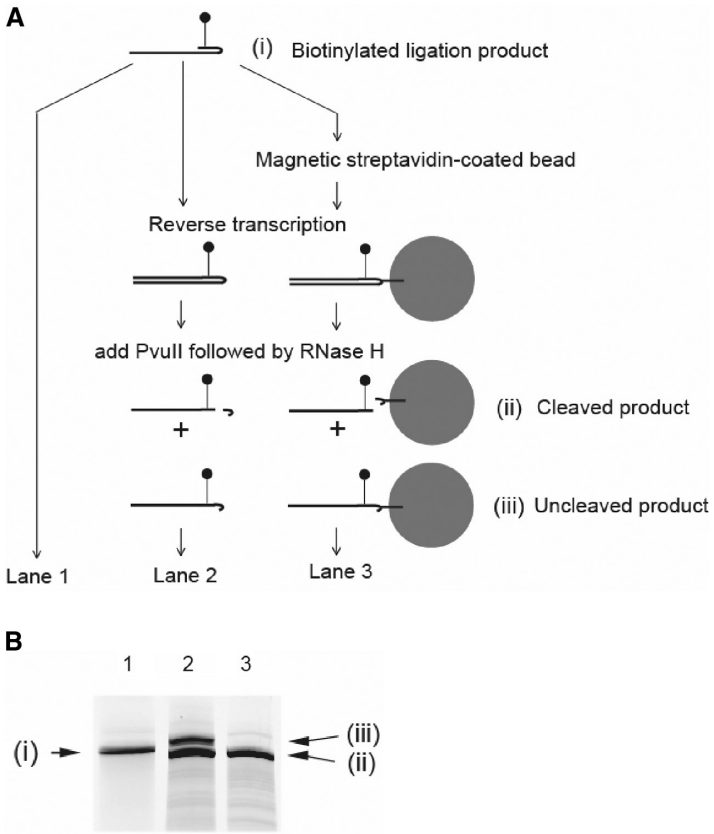

Figure 3. Solid-phase utilities of the puromycin–linker DNA. (**A**) Efficiency of release of the puromycin–linker DNA from the biotin moiety by PvuII cleavage. Schematic diagram of experiments to compare the efficiency of PvuII cleavage reaction in 'liquid phase' versus 'solid phase' approaches. An equal amount of the ligated products (mRNA–puromycin) were reverse transcribed in the liquid phase and on magnetic bead, respectively. RT products were then released from the biotin moiety by PvuII cleavage and from the mRNA portion by RNase H (20 U; 37°C, 20 min). (**B**) Efficiency of digestion by PvuII in the liquid phase (lane 2) and the solid phase (lane 3) was evaluated by 6% PAGE containing 8 M urea and detected by FITC fluorescence.

[Figure 5C(b)]. This shows that the BDA molecules are selected in a specific manner against IgG with a 20-fold higher efficiency than non-specific templates (i.e. PDO).

To further confirm the screening feasibility of our cDNA display method for another target, we performed a FLAG-tag selection against the anti-FLAG M2 antibody. A random DNA library and FLAG epitope

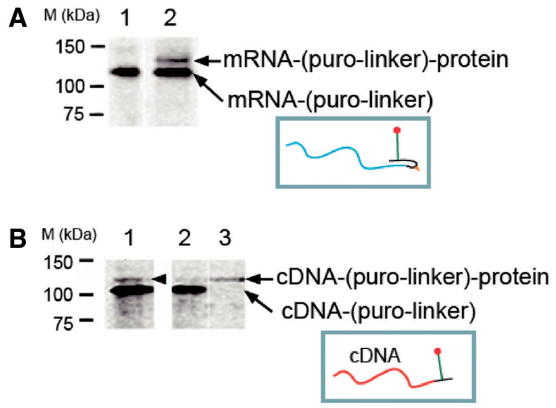

Figure 4. Efficiency of the formation of mRNA– and cDNA–protein fusion. (**A**) Synthesis of BDA mRNA–protein fusion in the liquid phase. mRNA–puromycin–linker with FITC (lane 1) was translated with a cell-free translation system followed by the addition of a high-salt solution (KCl and $MgCl_2$ to final concentrations of 750 and 63 mM, respectively) to the lysate to facilitate the formation of fusions via puromycin (lane 2) and evaluated by 6% SDS–PAGE containing 8 M urea and detected by FITC fluorescence. The efficiency of the fusion formation was calculated by using the following equation: % efficiency = $a/a + b \times 100$, where a is the band representing mRNA–linker–protein (upper band) and b represents mRNA–linker (lower band). (**B**) Synthesis of cDNA–protein fusion in the solid phase. BDA mRNA–puromycin–linker with FITC was immobilized on streptavidin beads followed by steps (d–i) as shown in Figure 1B. Lane 1, product after performing all the steps and digestion by RNase H (for detection of cDNA–linker and cDNA–linker–protein); lane 2, flow-through of the 6×-His purification process by Ni–NTA resin chromatography followed by RNase H treatment, and lane 3, eluate of the 6×-His purification process digested by RNase H. Amount in lanes 2 and 3 have been adjusted to contain 50 times the amount that was loaded in lane 1. The samples were evaluated by 6% SDS–PAGE containing 8 M urea and detected by FITC fluorescence. The efficiency of the formation of the final cDNA–linker protein was calculated based on the input template concentration (not shown here) in two steps. First, we calculated the efficiency of the formation of mRNA–linker–protein fusion using mRNA–linker templates (explained above). Second, we compared the intensities of the fusion bands (upper band) by taking into account the folds of cDNA–linker loaded (in this case 50). Therefore, % efficiency of formation of cDNA–linker–protein (Z) = $c/d \times f_{cDNA}$, where c is the mRNA–linker–protein, d is the cDNA–linker–protein and is folds of cDNA–linker loaded. The final efficiency of the formation of cDNA was calculated using the following equation:% efficiency = $[a/(a + b) \times 100]/Z$.

(DYKDDDDK)-encoding DNA templates were constructed as shown in Figure 5D(a). By using the initial library comprising 99.9% random DNA sequences and 0.1% FLAG DNA template, we examined whether the FLAG DNA template could be successfully amplified after several rounds of selection. The selected library pool after each round was analyzed by direct sequencing of the PCR products, and a convergence on the FLAG epitope-like sequence was evident after the third round of selection [Figure 5D(b)]. We also found that 17% of the total selected clones harbored the FLAG-encoded sequence in the third round, up from 0.1% at the start of the screening. These results indicate that cDNA display is indeed a useful and suitable method for the affinity selection of ligand peptides and functional proteins.

Selection of multiple disulfide bond containing molecules from a random library

To evaluate the potential performance of the method, we carried out selections using a 32-residue random library against IL-6R. We were interested in isolating peptides that were rich in cysteine residues and bonded by intramolecular disulfide bonds. For this purpose, we placed a fixed cysteine residue that preceded the 32 random residues with the expectation that cysteine residue(s) from the random region would be disulfide bonded with the fixed cysteine residue and selected. Affinity selection was carried out from rounds 1–3 (R1-R3) (see 'Selection of random library against IL-6R' section). After this, two selection schemes were designed (Figure 6A). In the first scheme, from R4 random, mutagenesis was performed during the PCR amplification (mRNA display-like procedure), while in the second scheme, from R4, in addition to mutagenesis, 'on-beads'-post-translational modifications were introduced before selection such as refolding in the presence of protein disulfide isomerase (PDI) (cDNA display-like procedure). After selection, both of the pools were cloned and sequenced.

The initial random library contained 32 random residues flanked by a fixed cysteine residue at the N-terminal and G_3S, 6×-His and G_2S at the C-terminal (Figure 6B). The selection using Scheme 1 enriched Cys-2-like sequences (12 out of 12 clones) after 12 rounds of selection, in which the two cysteine residues (i.e. one fixed cysteine residue and the second residue from the random region) were expected to be linked by one disulfide bond. In Scheme 2, the enriched sequences contained a mixture of Cys-2- and Cys-4-like sequences at 50% each (6 clones of each type out of the total 12 clones) after nine rounds of selection, in which two and four cysteine residues (i.e. one fixed cysteine residue and the other residues from the random region) were linked by one and two disulfide bonds. After R7, 30% of Cys-4 sequences were found among the selected clones compared to 50% at R9 that indicate that Cys-4 sequences were enriched over Cys-2 sequences (data not shown). Point mutations were observed within the sequences and could not be assigned to any group as no two sequences were identical (Figure 6B). Some of the selected candidates, from each selection scheme, containing two and four cysteine residues were chemically synthesized and were found to bind to IL-6R in the nanomolar range (4–100 nM; Table 1).

Due to the presence of four cysteine residues in Cys-4 peptides, various permutations were possible for the disulfide bond formation. To investigate the correct pairs of cysteine residues involved in the formation of disulfide bonds, we synthesized two different types of molecules (Cys4-2-A and Cys4-2-B) as shown in Figure 7A. In Cys4-2-A, disulfide bonds were formed between C1 and C4 and C2 and C3, while in Cys4-2-B, disulfide bonds were between C1 and C3 and C2 and C4, respectively. The two molecules were subjected to *in vitro* binding analysis for IL-6R. Indeed, we found that the pattern of disulfide bonds is important for the proper function. Cys4-2-A binds strongly to IL-6R, but Cys4-2-B did not show any apparent affinity (Figure 7B).

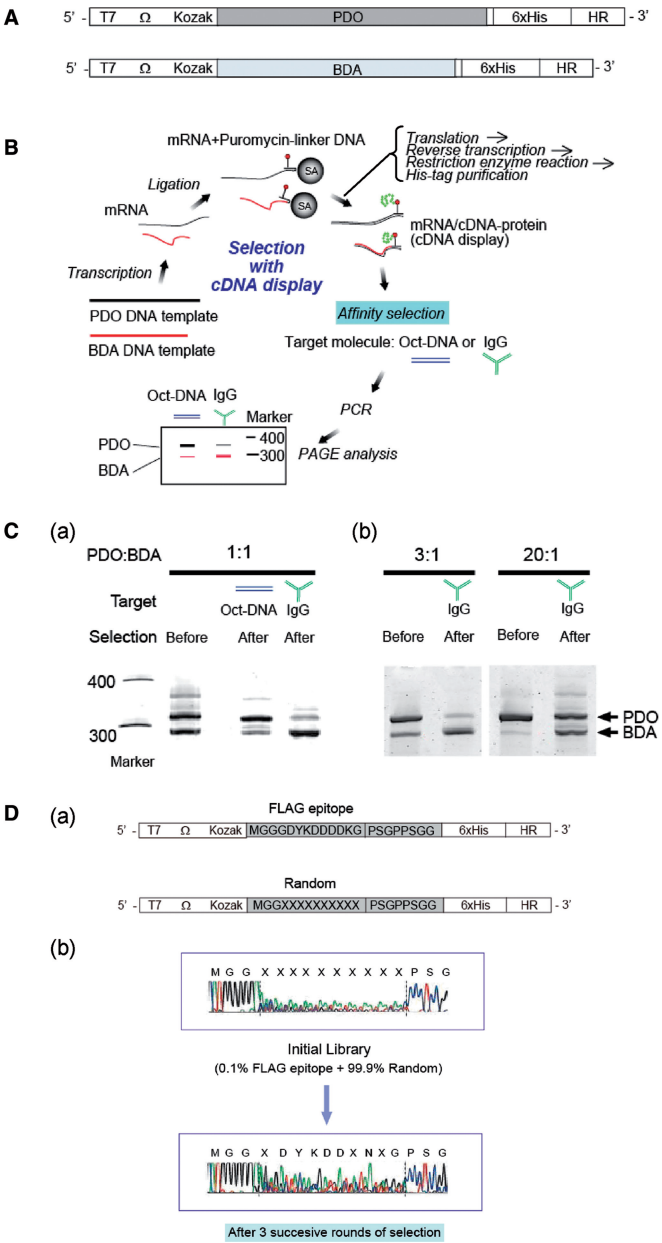

Figure 5. Validation of the cDNA display method by affinity selection experiments of proteins without disulfide bonds. (**A**) DNA template constructs used in the test screening experiments encoding the Pou-specific DNA-binding domain of Oct-1 (PDO) (top) or the B domain of protein A (BDA) (bottom). Ω is the 5′ untranslated region of tobacco mosaic virus and the HR region is a complementary puromycin–linker DNA sequence. (**B**) Selection scheme using cDNA display. PDO or BDA were used as templates to generate mRNA/cDNA–protein fusion products (cDNA display molecules) following the steps of transcription, ligation of mRNA to puromycin–linker, immobilization on streptavidin-coated beads, translation, fusion formation with mRNA, reverse transcription, restriction digestion and purification via 6×-His. The cDNA displayed mixture of both molecules was subjected to affinity selection against either Oct-DNA or IgG. The cDNA moieties of the selected molecules were then amplified by PCR from the eluted fraction after several washes and then analyzed by 8 M urea-denatured 5% PAGE. Selection against IgG enriches BDA (lower red band), while selection against Oct-DNA enriches PDO (upper black band). (**C**) The results of the affinity selection by the Oct-DNA or IgG. The initial molar ratio of PDO and BDA in the cDNA-display binary mixtures was 1:1 (a), 3:1 or 20:1 (b). After a single affinity selection against the given targets, the PCR products of the cDNA display molecules 'before' and 'after' selection were analyzed by 8 M urea-denatured 4.5% PAGE. The PDO and BDA are shown by arrows. (**D**) Model experiment of affinity selection of FLAG epitope by an anti-FLAG antibody. (a) The DNA encoding randomized 10 amino-acid residues and FLAG epitope (DYKDDDDK) were prepared as described in 'vectors, oligonucleotides and library construction'. The initial DNA library comprised a mixture of 1 pmol 'random' DNA templates and 1 fmol 'FLAG' DNA templates. Thus, the initial random peptide library contained 0.1% FLAG epitope. (b) Direct sequencing of the PCR products from the initial library and from the third round of affinity selection by a FLAG antibody.

DISCUSSION

Although it has been 10 years since the development of the *in vitro* virus and mRNA display (5,6), these techniques have not yet found common usage compared to the phage display (1). One of the reasons is the difficulty in preparing mRNA–puromycin conjugates (template) for translation in the mRNA–protein fusion reaction. Previously, large amounts of high-cost puromycin–linker DNA (at about a 200-fold molar excess over the input mRNA) were required because the efficiency of the ligation reaction was low due to the absence of hybridization between mRNA and the puromycin–linker (22). To overcome this problem, two methods have been previously reported: splint ligation and photo-crosslinked ligation. Using a splint oligo in order to reduce the ligation reaction time and excess amounts of puromycin–linker DNA, however, requires a quite cumbersome denaturing gel purification step to separate the splint-oligo DNA from the ligated mRNA template and this necessarily leads to the reduced recovery of the ligated product (23). The mRNA–puromycin conjugation by psoralen photo-crosslinking is rapid and efficient; however, mRNA degradation can occur as a result of the UV irradiation step during the process (24). On the other hand, ligation of mRNA and puromycin–linker by utilizing 'Y-ligation method' that involves T4 RNA ligase (25) is extremely fast under moderate conditions (25°C, ∼1 h) and thus avoids any damage to the mRNA by UV.

Another important aspect of mRNA display is its stability. A simple solution to improve stability is to convert the mRNA portion to a cDNA. Thus, the concept of cDNA–protein fusions has been previously proposed (12,13). In general, to perform *in vitro* protein selection using a cell-free translation system, double strand formation (i.e. mRNA/cDNA–protein fusion) would be important not only for the stabilization of the fusion molecule but also in order to restrict any unexpected interactions of the mRNA, which often binds to various kinds of biological molecules as aptamers or complementary oligonucleotides. However, it is very difficult to perform RT in a cell-free translation mixture because ribosomes are still bound to the mRNA and can disrupt the synthesis of the cDNA. We found that a combination of a rapid purification with streptavidin-coated magnetic beads under moderate conditions, buffer exchange and a rapid on-beads RT were very suitable for this purpose and

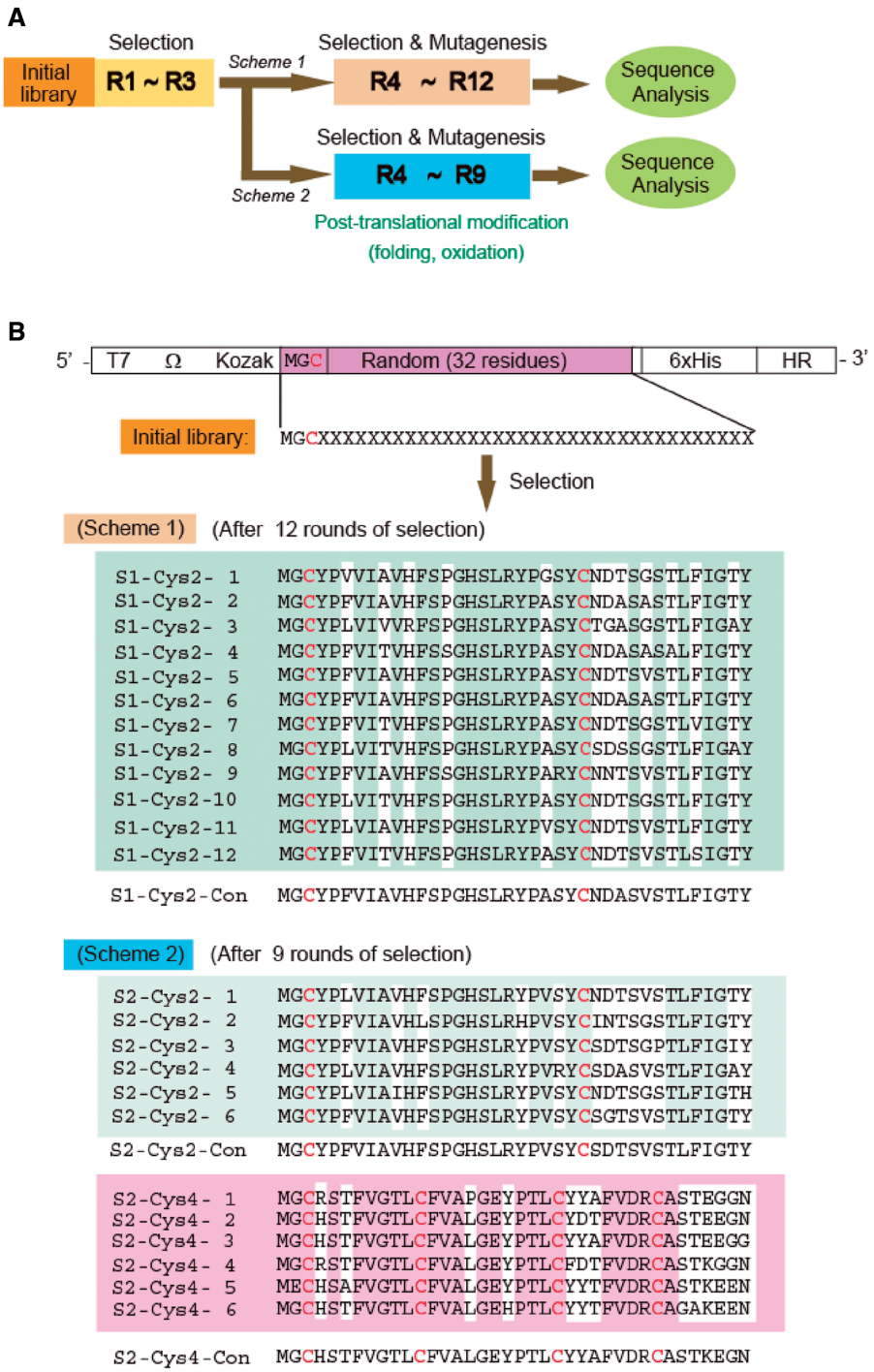

Figure 6. Selection of IL-6R binding molecules from a random library. (**A**) Selection schemes for the enrichment of disulfide bond-containing molecules. The initial library was subjected to affinity selection for three rounds (R1–R3). In Scheme 1 (mRNA display-like procedure), from R4 onward, random mutagenesis was introduced in the PCR amplification step of the R3 selected molecules followed by selection upto R12. In Scheme 2 (cDNA display-like procedure), in addition to random mutagenesis, post-translational modifications were also introduced and the peptides were allowed to fold in the presence of protein disulfide isomerase (PDI) and redox conditions followed by selection upto R9. The displayed peptides were purified owing to the ease of buffer exchange in solid-phase handling. The enriched pools from both the schemes were cloned and sequenced. (**B**) Library construction and sequence characteristics of the selected molecules. A random library was constructed containing 32 residues (shown by X), a fixed cysteine at the N-terminus and 6×-His at the C-terminus for the purification of the peptides. The library was subjected to affinity selection using the two schemes described in (A). Selection with Scheme 1 generated molecules containing two cysteine residues (denoted as Cys-2)—one fixed cysteine and the second from the random region (shown in red). The consensus sequence is shown as S1-Cys2-con and point mutations are highlighted in white. In Scheme 2, molecules containing two (Cys-2; top) and four cysteine residues (Cys-4; bottom) were enriched. S2-Cys2-con and S2-Cys4-con are the consensus sequences of Cys2 and Cys4 peptides, respectively. Point mutations are highlighted in white.

Table 1. Characteristics of the selected peptides

Peptides[a]	Sequence[b]	Selection scheme	Disulfide bonds[c]	K_d (nM)[d]
S1-Cys2-Con	CYPFVIAVHFSPGHSLRYPASYCNDASVSTLFIGTY	–	–	–
Sl-Cys2-1	CYPVVIAVHFSPGHSLRYPGSYCNDTSGSTLFIGTY	1	1 (C1-C2)	12 ± 1.4
Sl-Cys2-6	CYPFVIAVHFSPGHSLRYPASYCNDASASTLFIGTY	1	1 (C1-C2)	4 ± 0.6
Sl-Cys2-ll	CYPLVIAVHFSPGHSLRYPVSYCNDTSVSTLFIGTY	1	1 (C1-C2)	100 ± 9
S2-Cys4-Con	CHSTFVGTLCFVALGEYPTLCYYAFVDRCASTEEGN	–	–	–
S2-Cys4-2	CHSTFVGTLCFVALGEYPTLCYDTFVDRCASTEEGN	2	2 (C1-C4, G2-C3)	55 ± 3.7
S2-Cys4-3	CHSTFVGTLCFVALGEYPTLCYYAFVDRCASTEEGG	2	2 (C1-C4, C2-G3)	100 ± 12.5

[a]S1-Cys2-Con, and S2-Cys4-Con, are the consensus sequences of Cys2 and Cys4 candidates, respectively.
[b]Sequences of the random region (32 residues) are shown. Point mutations are indicated in bold. Cysteine residues are underlined.
[c]Disulfide bonds between the cysteine residues are indicated and were obtained by chemical synthesis. The importance of disulfide bonds for activity of the peptides was also investigated by preparing DTT reduced samples.
[d]These values were measured by Enzyme Linked Immuno Soibant Assay (ELISA; see Materials and Methods section).

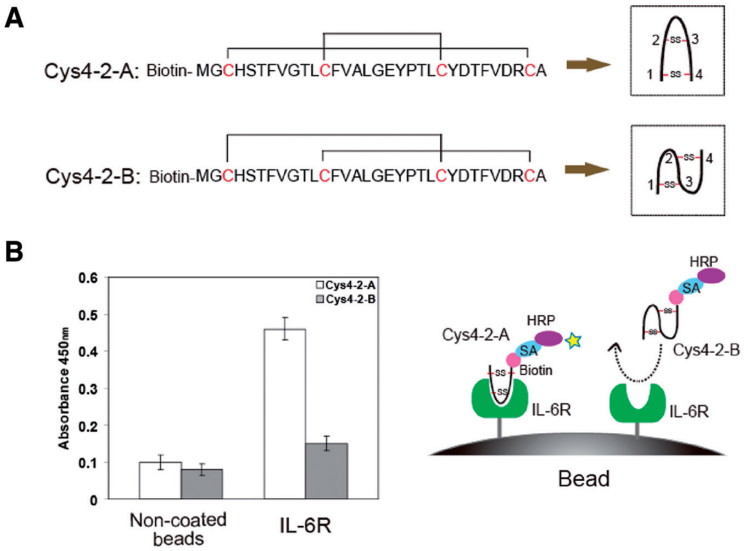

Figure 7. Possibilities of disulfide bond formation and the activity of the peptides. (**A**) Two possibilities out of various permutations for the disulfide bond formation were designed and tested. Cys4-2-A denotes clone 2 of S2-Cys4 series containing Type-A disulfide bond pattern i.e. C1 and C4, C2 and C3, while Cys4-2-B denotes clone 2 with disulfide pattern i.e. C1 and C3, C2 and C4. The schematic structures are shown on the right. (**B**) Biochemical binding assay. An *in vitro* biochemical assay based on was designed as shown in the schematic on the right-hand side. The biotinylated peptides were allowed to bind to IL-6R followed by binding of SA-HRP and detection with the addition of substrate. Non-coated beads denote beads without IL-6R. Cys4-2-A was found to bind to IL-6R and Cys4-2-B could not bind (also shown in the schematic on the right-hand side).

successfully reduced the time for RT to only 5 min compared to 30–60 min in the previous reports (12,13) (Figure 2). In this regard, the magnetic bead separation method by biotin–streptavidin chemistry is highly suitable in comparison with the oligo(dT) purification method that demands the undesirable elution conditions (the low salt buffer and high temperature) causing severe damage to the protein components. In our method, after RT, the subsequent release of immobilized cDNA fusion from the streptavidin beads by PvuII digestion is carried out at 37°C that is physiologically favorable and highly efficient (Figure 3). In general, PvuII cleaves only dsDNA of the linker DNA portion, but not of the mRNA/cDNA portion including random region. Thus, the diversity of a random library is not affected by the digestion reaction.

The performance of cDNA display in model experiments using ligand proteins was evaluated and found to be comparable to or better than the other methods (Figure 5). Therefore, we investigated the performance of our method in generating disulfide bond containing peptides from a random library as discussed below.

In the last decade, a minimizing antibody by rearranging antibody fragments (e.g. scFv) has been developed to overcome some problems of antibodies for clinical therapeutics. Recently, various kinds of scaffold derived from non-immunoglobulin proteins have been shown to be useful as alternatives of antibodies. In particular, these proteins offer smaller size for production and clinical effectiveness (26). Peptide scaffolds such as Kunitz domains, scorpion toxins, knottins and peptide aptamers are attractive for the development of lead molecules as drugs because of their small size (~40 residues) that can be synthesized and modified chemically. These peptide scaffolds have two or three disulfide bonds to stabilize

the structure compactly (27). However, when selections are performed for disulfide bond-containing peptide scaffold library by mRNA display, it may be difficult and impractical to display such proteins and/or libraries. Furthermore, refolding of proteins is also impractical as methods such as dialysis are tedious, time consuming and lead to low recovery of proteins. On the other hand, we have found that the foldability of the proteins synthesized on the solid surface using a cell-free translation system is increased with our method (14). Furthermore, proteins that are difficult to express can be displayed by combining solid-phase synthesis, easy buffer exchange and refolding using the puromycin–linker, which is the most important point. Based on solid-phase handling and selection from a 32-residue random library against IL-6R facilitated by cDNA display (Scheme 2 in Figure 6), we isolated peptides containing two disulfide bonds that were found to have a specific disulfide pattern for their activity (Figures 6 and 7 and Table 1). In addition, peptides containing a single disulfide bond could also be selected as in the case of mRNA display-like procedure (Scheme 1 in Figure 6; Table 1). Selection of peptides from a random library containing single disulfide bond have been reported (28); however, there are no published reports regarding selected peptides containing more than one disulfide bond. Therefore, this is the first report to select peptides containing two disulfide bonds from a random library by *in vitro* protein selection using cell-free translation. We believe that this will open up the new horizon to screen various kinds of functional proteins, which demands disulfide shuffling. Thus, cDNA display is becoming a far more efficient technique for functional protein screening compared to other reported display methods involving a liquid-phase approach.

In conclusion, novel and sophisticated cDNA display methods that make use of novel linkers such as ours will provide valuable new tools and solutions for the easier and more efficient *in vitro* selection of functional proteins using cell-free translation systems (Figure 1). This will also allow the use of various selection conditions with rapid, robust and stable mRNA/cDNA–protein fusions.

ACKNOWLEDGEMENTS

The authors are very grateful to Mr Toshikatsu Kobayashi for continuous support on this work, Professor Koichi Nishigaki and Dr Miho Suzuki for their valuable suggestions and discussions and Dr Zeev Pancer for critical comments on the manuscript. They also thank Dr Tomomi Toda and Dr Shinsaku Ohtaki for cooperation and Takahiro Mitsui and Sachika Tsuji for technical assistance.

FUNDING

This research was supported by grants from the Rational Evolutionary Design of Advanced Biomolecules (REDS) Project, Saitama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence (CREATE) and Core Research for Evolutional Science & Technology (CREST) from Japan Science and Technology Agency (JST), Strategic Research Center Development Program of Special Coordination Funds for Promoting Science of MEXT (Super COE), the City Area Program of Saitama Metropolitan Area from Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) and Innovation Center for Start-Ups, National Institute of Advanced Industrial Science and Technology (AIST)-Japan. Funding for open access charge: CREST, Japan

Conflict of interest statement. None declared.

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