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A Novel Strategy by the Action of Ricin that Connects Phenotype and Genotype without Loss of the Diversity of Libraries

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Abstract: We present a novel strategy for connection of phenotype and genotype in vitro that can be used for the selection of functional proteins even at room temperature. The strategy involves generation of a stable complex between a ribosome, an mRNA, and its translated protein, without removal of the termination codon, as a result of the action of the ricin A chain during translation. We demonstrate the potential selection capacity of this novel strategy by isolating such complexes that contain newly synthesized streptavidin and glutathione-*S*-transferase (GST) using appropriate ligands. The technique requires no transfection, no chemical synthesis, no ligation, and no removal of the termination codon. Thus our novel "Ribosome-Inactivation Display System (RIDS)" should provide, without loss of the pool population, a reliable, simple, and robust selection system for in vitro evolution of the properties of proteins in a predictable direction by a combination of randomization and appropriate selection strategies.

Introduction

During the past decade, several display strategies have provided powerful and efficient techniques for the selection and evolution of peptides and proteins. In these techniques, the coupling of genotype and phenotype is the single most critical determinant in the selection of functional proteins. In such selection systems, the specific sequence information (genotype) of members of libraries that encode the selected protein (phenotype) can be determined from the corresponding DNA/RNA that was introduced into the system. The gene encoding the selected protein can then be re-amplified for further evolution and analysis. The strategies that have been successfully developed are either cell-dependent (Figure 1), involving, for example, display on the surface of phage,[1] other viruses,[2] bacteria[3] or yeast,[4] or they are cell-free, as in the case of ribosome display[5−7] and mRNA display[8,9] systems.

The methods that have been developed to date are, however, associated with certain limitations and disadvantages. Since cell-dependent display systems[1−4] include a necessary in vivo step, the sizes and diversity of sequence libraries are limited by the efficiency of transformation and by the nature of the protein in question. For example, some proteins that are detrimental to cells or that have important regulatory functions within cells cannot be selected. An alternative method, namely, cell-free ribosome display,[5−7] must be adopted at a low temperature under conditions that preserve the integrity of the protein-ribosome-mRNA ternary complex, with removal of a termination codon and the addition of magnesium acetate and anti-*ssrA* oligonucleotides.[6,7] Moreover, the yield of the isolated mRNAs after one round of ribosome display is not very high. In fact, it can be as low as 0.015% of the input RNA, with a resultant potential reduction in the diversity of the sequence libraries. The alternative cell-free, mRNA display procedure[8,9] requires careful chemical synthesis and critical purification of puromycin-attached oligonucleotides, which must be ligated to the 3′ end of each mRNA in the sequence libraries. Failure to perform these manipulations appropriately leads to a reduction in the diversity of available libraries.

To solve the above-mentioned problems and to maintain the diversity of sequence libraries, we developed a new strategy that allows us to prepare a protein-ribosome-mRNA ternary complex that is significantly more stable than that obtained by

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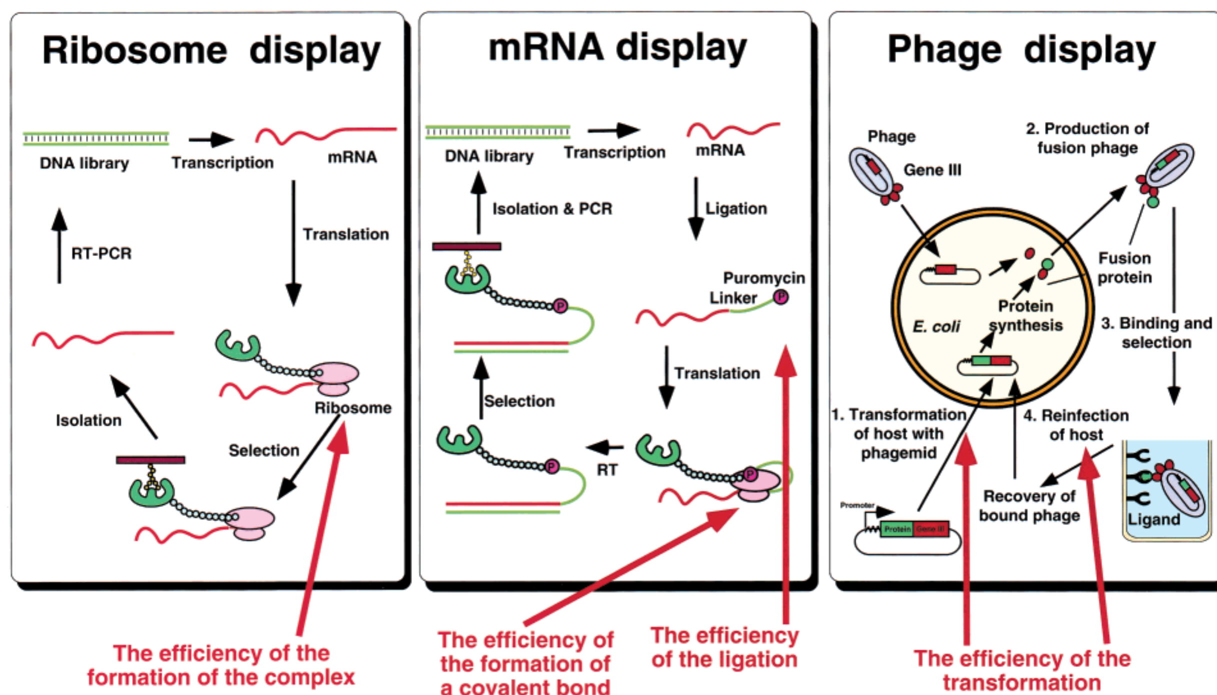

Figure 1. Schematic diagrams of representative display systems with identification of the steps in each that tend to reduce the diversity of the randomized libraries. Efficiencies of the ribosome complex, of the chemical synthesis of puromycin-attached oligonucleotides and their ligation to mRNAs, of the formation of the covalent bond with a nascent peptide via puromycin, and of the transformation reduce the size of the library. The recent novel cell-free protein-synthesizing system, reconstituted with pure components, might also be useful for the ribosome display.[44]

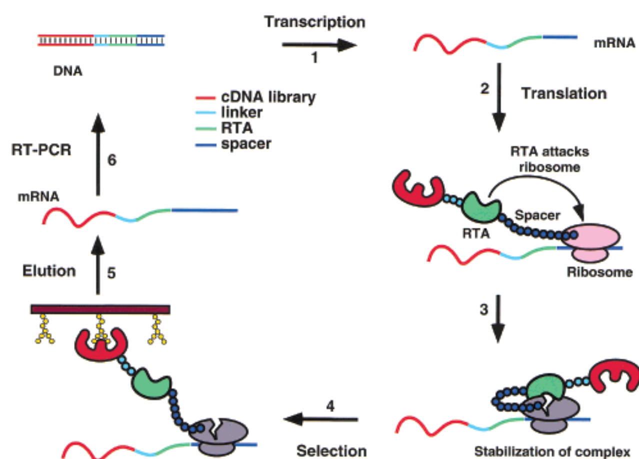

Figure 2. Schematic representation of the Ribosome-Inactivation Display System (RIDS) for screening functional proteins in vitro. Step 1: The gene for the ricin A chain (RTA), inserted downstream of a random protein library (or cDNA library), is transcribed by T7 RNA polymerase to yield mRNA. Step 2: mRNA is translated in a rabbit reticulocyte lysate system. Step 3: During translation, since the rRNA is inactivated by folded RTA in a cis reaction, the ribosome is stalled and a ribosome−mRNA−protein complex is formed. Step 4: The complex of interest is bound to the corresponding affinity matrix. Unwanted complexes are removed by washing. Step 5: The specific complex is dissociated from the matrix by elution with a buffer that contains EDTA and then free mRNA is isolated. Step 6: Eluted RNA is amplified by RT-PCR and the resultant cDNA is used for the next cycle or for analysis by cloning and sequencing.

the conventional method, even at room temperature, without reducing the diversity of the library pool. Stabilization was achieved by introducing the gene for a toxin that inactivates eukaryotic ribosomes, the ricin A chain (RTA; indicated in green in Figure 2), downstream of the region of the sequence library that would be translated into a protein library (indicated in red in Figure 2). Ricin, which is composed of A and B chains, is a plant toxin that inhibits protein synthesis by inactivating ribosomes. The B chain is required for the internalization of ricin into cells and the RTA, which is the active moiety, catalyzes the hydrolysis of a specific *N*-glycosidic bond adjacent to the universally conserved adenosine that is found in a GAGA tetra-loop in 23S-28S rRNAs in the large subunit of eukaryotic ribosomes.[10,11] This single depurination, which alters the binding site for elongation factors, inhibits protein synthesis and stalls ribosomes on the translation complex without release of the mRNA or the translated protein.[12−14] Translation is stalled before the termination codon has been reached and, thus, the recruitment of release factors to the ribosome does not occur. Consequently, the mRNA and the nascent protein remain bound to the ribosome very stably, and not just for hours but for days. In particular, since RTA is able to inactivate approximately 1500 ribosomes/min,[15] extremely stable protein-ribosome-mRNA complexes are rapidly formed at room temperature under standard translation conditions. Therefore, a key aspect of our "Ribosome-Inactivation Display System" (RIDS) is that the ribosome is stalled on an mRNA via an entirely different mechanism from that of the original ribosome display system.

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Using this method, we should be able to screen functional proteins without removing the termination codon under standard translation conditions.

To evaluate the potential utility of RIDS, we chose streptavidin and glutathione-*S*-transferase (GST) as target proteins and isolated the respective proteins and their mRNAs using appropriate matrices that contained biotin and glutathione, respectively, as the ligands. We found that it was possible, using RIDS, to screen the functional proteins (streptavidin and GST) without reducing the diversity of the sequence library without any chemical synthesis. To the best of our knowledge, RIDS, which "rids" experiments of problems associated with loss of diversity of the sequence library, provides the simplest and most robust strategy for selection of functional proteins.

Results and Discussion

Rationale for the Ribosome-Inactivation Display System (RIDS). In RIDS, to avoid the limitations and disadvantages of cell-dependent or cell-free display methods, we introduced the genetic sequence that encodes the ribosome-inactivating protein RTA at the 3′-terminal end of all members of a random sequence library (or cDNA library) so that, upon completion of the synthesis of each library protein, which would be directly linked to RTA, the nascent RTA would rapidly inactivate the ribosome and the protein-ribosome-mRNA ternary complex would remain stable indefinitely under the conditions of the translation reaction (Figure 2). Transcripts of the DNA sequence library were translated in a rabbit reticulocyte lysate. After translation, we anticipated that the proteins would fold naturally and RTA, fused to the carboxyl terminus of each protein in the library, would recognize and hydrolyze the *N*-glycosidic bond at the key purine residue of rRNA in the ribosome and stop translation immediately. The stable maintenance of the protein-ribosome-mRNA complex would allow us to connect phenotype and genotype. Using the appropriate immobilized affinity ligand or antigen, we postulated that we would then be able to capture the protein-ribosome-mRNA complex that would incorporate both the phenotype and the genotype, simultaneously. After dissociation of the selected complex, we would be able to amplify the eluted mRNA by RT-PCR for subsequent cycles or for further evolution and analysis.

Construction of RIDS. Our goal was to establish a simple and robust protein-selection system in vitro. To confirm the validity of the proposed method and the potential utility of RIDS, we introduced the gene for streptavidin or GST into a DNA sequence library (Figure 3). These proteins have frequently been fused to newly discovered proteins and/or the molecules with which they interact for the successful functional characterization of such newly discovered, fused proteins.[16−19] Streptavidin and GST bind to biotin and glutathione, respectively, with high affinity and specificity. Thus, it should be possible to isolate and characterize the protein-ribosome-mRNA ternary complex with ease, as shown in Figure 2. To eliminate steric hindrance between streptavidin or GST and the downstream RTA, a flexible linker was indispensable that would allow nascent proteins to fold into their natural three-dimensional structures. Previous investigations demonstrated that the yield of a selected protein/mRNA is strongly dependent on the length, composition, and sequence of such a linker.[6,20,21] In this study, we used a Gly/Ser-rich fragment of 47 amino acids as the linker.

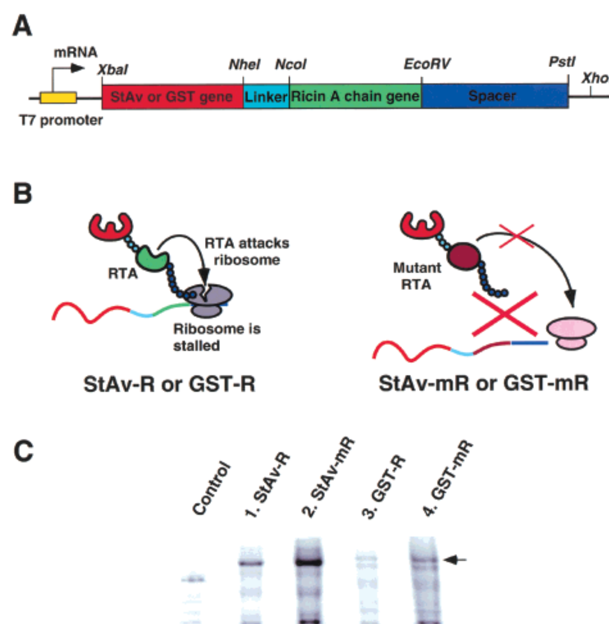

Figure 3. (A) The DNA construct used for RIDS. A T7 promoter is followed by the gene for streptavidin-RTA or GST-RTA fusion protein. A glycine/serine-rich linker is located between the gene for the target protein and the gene for RTA to eliminate steric hindrance between the protein of interest and RTA after translation. A spacer is inserted downstream of the gene for RTA to ensure the activity of RTA. Four plasmids of this type (pStAv-R, pGST-R, pStAv-mR, and pGST-mR) were constructed. (B) StAv-R and GST-R fusion proteins stall ribosomes, with resultant formation of stable complexes. StAv-mR and GST-mR fusion proteins cannot stall ribosomes and do not yield stable complexes because RTA has been mutated to an inactive form (mR). (C) Analysis by SDS-PAGE of the products of in vitro translation in a rabbit reticulocyte lysate system. Proteins were labeled with ^{35}S-methionine. Control, Luciferase, as a standard (61 kDa); lane 1, StAv-R fusion protein; lane 2, StAv-mR fusion protein; lane 3, GST-R fusion protein; lane 4, GST-mR fusion protein. The yields of proteins were significantly higher when the mutant RTA was generated because mutant RTA failed to inactivate ribosomes as shown schematically in part B (right).

In addition to the linker, a spacer at the 3′-terminal end of the open reading frame (ORF) was also important. The spacer, which was required as an anchor to tether the ribosome, must be of appropriate length so that it can occupy the long tunnel of the ribosome[22,23] and allow the nascent RTA to fold correctly without any steric hindrance.[24,25] It has been reported that a spacer of at least 20−30 amino acids at the carboxyl terminus is required to retain the activity of an enzyme displayed on a ribosome.[26,27] Thus, the amount of mRNA isolated by the ribosome display system is influenced by the length of the spacer

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and the secondary structure of its end.[6,22,28] We introduced a long and a short spacer sequence, separately, at the 3′-terminal end of the ORF and compared the effects of the two spacers on translation and selection. We found that the long spacer (of 404 amino acids, encoded by gene III, in its entirety, from the filamentous phage M13) was not suitable for translation and selection in our system (data not shown). Therefore, in all our experiments, we used a fragment of 120 amino acids as the spacer and anchor for the streptavidin-based and GST-based template plasmids, pStAv-R (StAv, streptavidin; R, ricin A chain) and pGST-R, and the corresponding inactive variants, pStAv-mR and pGST-mR (mR, mutated inactive ricin A chain; see below).

Inhibition of Translation by RTA. To confirm that RTA inhibits translation and helps to maintain a stable ribosome complex, in a control experiment, we made a gene for an inactive, mutant RTA by site-specific mutagenesis, changing functional amino acids as follows: glutamic acid 177 to glutamine; arginine 180 to histidine; and glutamic acid 208 to aspartic acid. Mutation of these three amino acids in RTA completely abolished its activity.[29,30] Since mutant RTA cannot inactivate ribosomes (the mutant inactive RTA was encoded by plasmids pStAv-mR and pGST-mR), we postulated that, when ribosomes reached the termination codon after translation of the spacer, the termination codon would initiate the recruitment of release factors. Immediate release of the ribosome from the mRNA would make the ribosome available for another round of translation. Multiple turnover of ribosomes would result in further translation and generation of fusion proteins. No stable protein-ribosome-mRNA complex should be formed (Figure 3B, right).

We examined first whether RTA acted on inactivated ribosomes by comparing efficiencies of translation. Each template plasmid (pStAv-R, pStAv-mR, pGST-R, or pGST-mR) was transcribed by T7 RNA polymerase and then an equal amount of each mRNA was translated in rabbit reticulocyte lysates. The production of each protein was monitored by SDS-PAGE (Figure 3C). The production of fusion proteins that included the mutant RTA (lane 2, StAv-mR and lane 4, GST-mR) was significantly higher than that of the fusion proteins that included the wild-type RTA (lane 1, StAv-R and lane 3, GST-R). The small amounts of wild-type products suggested strongly that the RTA fusion molecules had really inhibited translation and that multiple turnover of ribosomes was significantly suppressed.

Selective Enrichment of RTA Fusion mRNA. The mRNAs encoding StAv-R and GST-R and the control mRNAs encoding mutant RTA (StAv-mR and GST-mR) were translated separately and reaction mixtures were exposed to affinity ligands (biotin-agarose or glutathione-Sepharose) to confirm that the mRNAs bound to the respective ligands through a complex between the ribosome and the protein. Translation with mRNAs that had been labeled internally by [α-^{32}P] CTP was examined to determine the amount of bound mRNA at each step during the selection procedure. After translation for 20 min, the appropriate binding buffer and ligand (namely, biotin-agarose or glutathione-Sepharose) were added to lysates and binding reactions were allowed to proceed for 30 min at room temperature. Each ligand had been blocked, before combination with a translation mixture, by exposure to sterilized milk (Block Ace; Dainippon Pharmaceutical Co., Japan) and each binding and washing step was performed in the presence of the milk since milk decreases the nonspecific binding of ribosome complexes to agarose.[31] After centrifugation, supernatants that contained unbound mRNA and protein were discarded and respective pelleted ligands were washed four times with binding buffer. At each step during the selection procedure, the relative amount of mRNA that remained in the mixture was determined by monitoring radioactivity. The mRNA and the nascent protein remain bound to the ribosome very stably for days (data not shown).

To examine the selection of a specific mRNA, we subjected StAv-R, GST-R, and StAv-mR mRNAs to binding analysis with biotin-agarose (Figure 4, parts A and B). GST-R and StAv-mR mRNAs were soon washed away completely, while StAv-R was firmly retained with more than 5% of the input mRNA remaining after the fourth wash (Figure 4A). It is important to note that the amount of recovered mRNA, which remained at 5% after repeated washes, was significantly higher than that isolated by a related ribosome display system, which was as little as 0.015% (or 0.2% under certain conditions) of the input RNA. The control mRNAs could be washed away effectively and only enrichment for the expected StAv-R occurred (Figure 4B).

To strengthen our findings, we subjected GST-R, StAv-R, and GST-mR mRNAs to binding analysis with glutathione-Sepharose. As anticipated, StAv-R and GST-mR mRNAs were soon washed away completely, while GST-R was firmly retained by the ligand (Figure 5, parts A and B). However, in this case, the amount of recovered mRNA after repeated washes was 1.3% of the input RNA. We experienced that, in many cases, because of unknown reasons, the recovery yields varied from >10% to <1%, depending on the preparation of the input RNA.

Our results demonstrated that (i) a protein-ribosome-mRNA ternary complex was formed when the fusion protein contained the active wild-type RTA, (ii) mRNAs bound to the appropriate ligand with high specificity, and (iii) the extent of nonspecific binding of mRNAs to unrelated ligands was very low.

If RTA is to function properly in the selection of a target protein, the protein in question should end up bound specifically to its mRNA when two distinguishable mRNAs are translated together. With this goal in mind, we attempted the enrichment of two mRNAs (StAv-R and GST-R) with each respective ligand using RIDS. Transcribed mRNAs were translated in a reticulocyte lysate either individually or as a mixture at a ratio of 1:1 (mole/mole) and respective mRNAs were selected on biotin-agarose or glutathione-Sepharose.[32,33] The binding and washing procedure was basically the same as that used in the experiments described above. We then eluted bound products from ligands with elution buffer in the presence of EDTA at 100 °C. The eluted mRNAs were purified with an RNeasy mini kit (QIAGEN, Germany) and amplified by RT-PCR to confirm the enrichment

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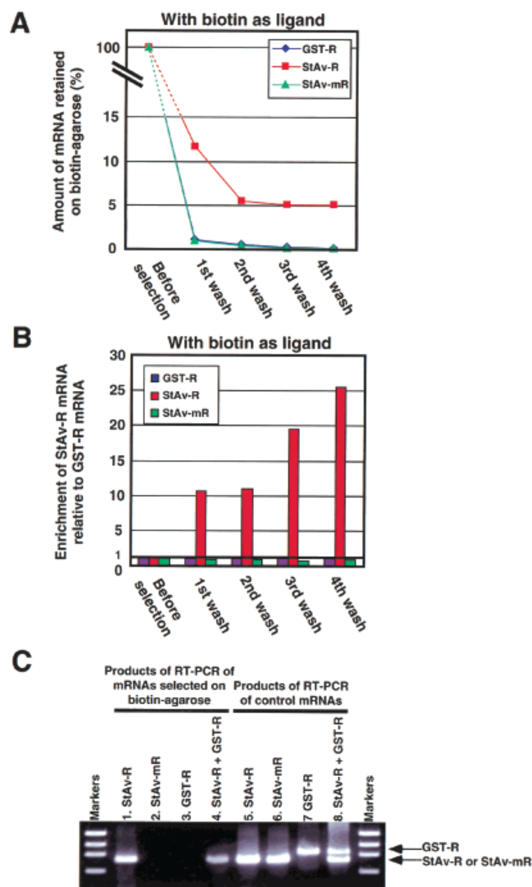

Figure 4. Analysis of the results of selection with biotin-agarose. (A) Binding of mRNA via the ribosome and the protein to biotin-agarose. mRNA was labeled with [α-^{32}P] CTP. (B) Enrichment for StAv-R mRNA by binding to biotin-agarose, as compared to GST-R and StAv-mR mRNAs. Only the streptavidin-containing complex bound specifically to biotin-agarose when the RTA was active. (C) Analysis by agarose gel electrophoresis of the results of amplification by RT-PCR. Markers, size markers; lanes 1−4, products of mRNAs selected with biotin-agarose; lanes 5−8, products of mRNAs before selection (without selection) as controls. Lanes 1 and 5, StAv-R; lanes 2 and 6, StAv-mR; lanes 3 and 7, GST-R; lanes 4 and 8, a mixture of StAv-R and GST-R at a ratio of 1:1 (mole/mole).

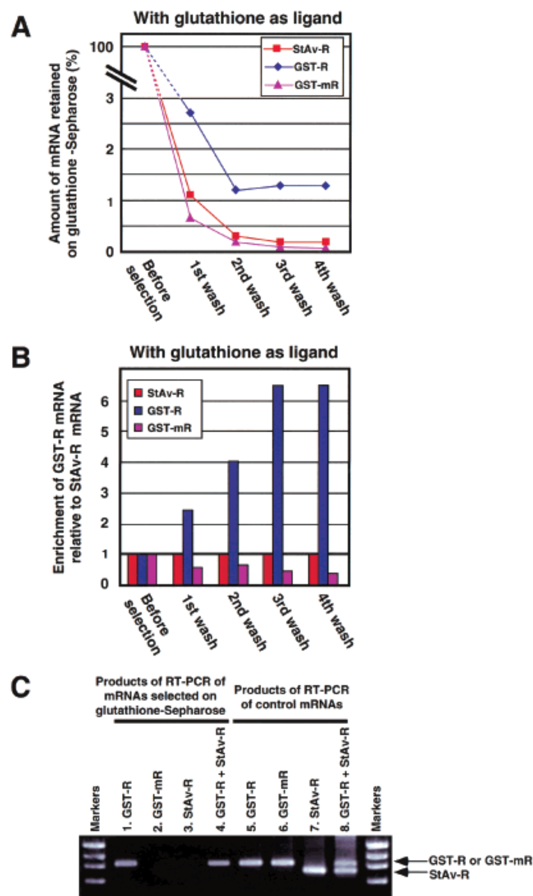

Figure 5. Analysis of the results of selection with glutathione-Sepharose. (A) Binding of mRNA via the ribosome and the protein to glutathione-Sepharose. mRNA was labeled with [α-^{32}P] CTP. (B) Enrichment for GST-R mRNA by binding to glutathione-Sepharose, as compared to StAv-R and GST-mR mRNAs. Only the glutathione-*S*-transferase-containing complex bound specifically to glutathione-Sepharose when the RTA was active. (C) Analysis by agarose gel electrophoresis of the products of RT-PCR. Markers, size markers; lanes 1−4, products of mRNAs selected with glutathione-Sepharose; lanes 5−8, products of mRNAs before selection (without selection) as controls. Lanes 1 and 5, GST-R; lanes 2 and 6, GST-mR; lanes 3 and 7, StAv-R; lanes 4 and 8, a mixture of StAv-R and GST-R at a ratio of 1:1 (mole/mole).

for the mRNA of interest (Figures 4C and 5C). We confirmed previously by PCR that each sample of mRNA did not contain any contaminating DNA (data not shown). Furthermore, we confirmed that the amount of each input mRNA was constant (Figures 4C and 5C, lanes 5−8). Our analyses by RT-PCR demonstrated clearly that mRNA encoding streptavidin or GST was successfully selected, when each was translated separately (corresponding to the experiments shown in Figures 4A and 4B and Figures 5A and 5B), by biotin-agarose or glutathione-Sepharose, respectively (Figures 4C and 5C, lane 1), confirming our earlier conclusions.

When we mixed the mRNAs that encoded streptavidin and GST together at a ratio of 1:1 (mole/mole), each was correctly concentrated from the mixture via binding to biotin-agarose and glutathione-Sepharose, respectively (Figures 4C and 5C, lane 4). By contrast, the nonspecific binding of unwanted mRNAs to the biotin and glutathione on matrices of agarose and Sepharose, respectively, was undetectable (Figures 4C and 5C, lanes 2−4), even though it has been reported that streptavidin and GST fusion proteins can generate false positive results in protein selection.[34−36] The active form of RTA was essential, in every case, for enrichment of the mRNA that encoded the target protein (Figures 4C and 5C, lane 2).

Conclusions

In this study, we developed a Ribosome-Inactivation Display System (RIDS) for selection of functional proteins in vitro and we confirmed the validity of our approach by the successful display of streptavidin and GST. In RIDS, the RTA fused to the carboxyl terminus of the target protein stalls the ribosome and a stable protein-ribosome-mRNA ternary complex is formed when translation is interrupted. In our system, the ribosome is stalled on an mRNA by a mechanism that is entirely different from the mechanism of the original ribosome display system. In conventional ribosome display, selection must be performed

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at a very low temperature to maintain the stability of the ternary ribosomal complex. This requirement might be the bottleneck for some selections that require higher temperature.[37,38] By contrast, in RIDS, the ternary complexes are very stable, even at room temperature, and removal of the termination codon and special conditions, such as addition of magnesium acetate and anti-*ssrA* oligonucleotides, are not required. Our method is obviously better for experiments in which rare functional proteins are to be selected at higher temperatures. Our own data demonstrated that the selectivity increases at higher temperatures (unpublished data).

The in vitro evolution of functional ATP-binding proteins was recently achieved by selection from a library that contained 80 contiguous random amino acids in sequential rounds of mRNA display.[39] RIDS should complement such techniques as a simple and effective approach to mRNA-protein display for the selection of specific functional peptides or proteins since our system requires no chemical synthesis and does not include any steps that might potentially reduce the diversity of the pool of sequences. There have been several reports of studies aimed at the selection of human antibodies.[7,40,41] RIDS should be useful for the selection of any protein of interest for both diagnosis of human diseases and therapy because the proteins are selected in a "eukaryotic environment", namely, a rabbit reticulocyte lysate.

RIDS should be a very powerful strategy for the generation and evolution of new proteins since the amount of recovered mRNA is more than 2 orders of magnitude higher than that obtained in a conventional related system (Figures 4 and 5). Moreover, RIDS requires only the introduction of genes of a random protein library or cDNA library upstream of the gene for RTA for the selection of functional proteins. This simple yet high-yield system should contribute significantly to proteomics research in the post-genome era.[42]

Experimental Section

Construction of Plasmids. All constructs were confirmed by DNA sequencing and restriction mapping. The plasmids pStAv-R and pGST-R were constructed as follows: (i) DNAs coding for residues of streptavidin and GST was excised from plasmids pSTA (a gift from Prof. M. Sisido, Okayama University) and pGEX-4T-3 (Amersham Pharmacia Biotech. Sweden), respectively. These fragments were amplified by PCR and ligated separately into the *Xba*I/*Nhe*I site in modified pET30a (Novagen, Germany). (ii) DNA coding for the linker was amplified by PCR from the glycine/serine rich region of gene III of M13 and ligated to the 3′ terminus of the sequence that encoded streptavidin or GST by *Sca*I/*Eco*RV. (iii) DNA coding for RTA was prepared by PCR from pUTA (a gift from Prof. J. Robertus, University of Texas) and inserted downstream of the linker by *Nco*I/*Eco*RV. (iv) DNA coding for the spacer was derived from gene III of M13 and inserted downstream of RTA by *Eco*RV/*Pst*I.

Site-Directed Mutagenesis. To construct pStAv-mR and pGST-mR, the gene for RTA in each plasmid (StAv-R or GST-R) was replaced by a mutant gene for RTA by standard procedures[43] with the following primers: 5′-TTGCATCCAAATGATTTCACAAGCAGCACACTTCCAATATATTAGGGAGAAATG-3′ (underlining indicates mutations E177A and R180H) and 5′-GATCCTAGCGTAATTACACTTGACGATCCTAGCGTAATTACACTTGA-3′ (underlining indicates mutation E208D).

Transcription in Vitro. These various plasmids (pStAv-R, pGST-R, pStAv-mR, and pGST-mR) were digested in the 3′-terminal region of the ORF by *Xho*I to yield linear DNAs and then DNAs were transcribed with a T7 RNA transcription kit (Epicentre Technologies Co., U.S.A.) with a cap-structure analogue (New England Biolabs, England) to produce nonradioactive or radioactive mRNA. Then mRNAs were purified with an RNeasy mini kit (QIAGEN, Germany).

Translation in Vitro and SDS-PAGE. Transcripts were translated in vitro in a rabbit reticulocyte lysate system (Promega, U.S.A.). Translation was allowed to proceed for 1 h at 30 °C in a 25-μL reaction mixture that contained 20 μL of lysate, 0.5 μL of amino acid mixture minus methionine, 0.5 μL of RNase inhibitor, 3 μL of ^{35}S-methionine (NEN Life Science Products, Inc., U.S.A.), and 2.0 μg of template mRNA. After translation, proteins were denatured in loading buffer for SDS-PAGE at 100 °C for 3 min and were then analyzed by SDS-PAGE (7.5% polyacrylamide) for 1 h at 30 mA.

Affinity Selection of the Ribosome-mRNA-Protein Complex and Isolation of mRNA. Before selection, translation was allowed to proceed as described above with modifications: the reaction time was reduced to 20 min and we used 0.5 μL of a complete amino acids mixture and radiolabeled mRNAs. After incubation for 20 min, the reaction was stopped by placing each tube on ice for 5 min. Then, 25 μL of a suspension of biotin-agarose (Sigma Chemical Co.,U.S.A.) or glutathione-Sepharose (Amersham Pharmacia Biotech., Sweden) was added to each tube and finally 100 μL of binding buffer was added. The binding buffer for biotin-agarose contained 47 mM K_2HPO_4, 3 mM KH_2PO_4, 300 mM NaCl, 0.05% Tween, and 2% Block Ace at pH 7.0. The binding buffer for glutathione-Sepharose contained 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, 2.7 mM KCl, 140 mM NaCl, 1% Triton X-100, and 2% Block Ace at pH 7.4. After gentle rotation for 30 min at room temperature, each mixture was centrifuged at 500 $\times$ g for 2 min and the supernatant was removed. The pellet was washed four times with binding buffer. At each step during the selection procedure, the remaining relative amount of mRNA was monitored with a radioisotope counter (Bioscan, Inc., Washington, D.C.). After the fourth wash with binding buffer, the protein-ribosome-mRNA complexes retained on matrices were released by treatment with elution buffer (binding buffer plus 3 M guanidine and 250 mM EDTA). After denaturation by heating at 100 °C for 3 min, the eluted mRNA was recovered with an RNeasy kit. The purified RNA was used for reverse transcription and PCR.

Reverse Transcription and PCR. Reverse transcription was performed with AMV reverse transcriptase (Promega, U.S.A.) according to the supplier's recommendation. PCR was performed with Ex Taq polymerase (Takara, Japan; 2 min at 96 °C, followed by 25 cycles of 45 s at 96 °C, 90 s at 54 °C, and 2 min at 72 °C, with a final 10-min extension at 72 °C). Products of PCR were analyzed by electrophoresis on agarose gels.

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