論文の内容の要旨

論文題目

Development of tRNA-engineered *in vitro* translation systems to synthesize peptides with expanded repertoire of nonproteinogenic amino acids

(多種の非タンパク質性アミノ酸を基質として利用する tRNA 改変型ペプチド翻訳合成系の開発)

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Introduction

Proteins and peptides, which are collectively called as polypeptides, perform a vast array of biological functions and attract a great interest in biological and pharmaceutical sciences. In living organisms, polypeptides are synthesized by ribosomal translation reaction according to the sequence of codons that comprise three nucleotide units in mRNA sequence. The relationship between codons and amino acids is referred to as the genetic code (Fig. 1A). Among 64 codons, 61 codons designate elongation of the nascent polypeptides with specific proteinogenic amino acids, synthesizing amino acid polymer with 20 proteinogenic building blocks. Beyond this nature's limitation, artificial engineering of translation machinery has allowed for the ribosomal synthesis of polypeptides containing nonproteinogenic amino acids (npAAs)[1], which have created innovative and practical scientific fields. For example, the synthesis of polypeptides containing optical probes facilitates structural biology by NMR and X-ray crystallography, and also it contributes to the development of chemically modified "nonstandard" peptide drugs with improved pharmacological properties. In particular, our laboratory has developed a selection technology to discover potent peptide binders to drug target proteins, named RaPID (Random nonstandard Peptides Integrated Discovery) system, and successfully developed 'nonstandard' peptide drugs with noncanonical scaffolds that mimic the characteristics of naturally occurring bioactive peptides.

In spite of the supposed practicability, the current engineered translation systems still have methodological limitations that diminish its scope of application: (1) Accurate synthesis of a polypeptide containing more than two

distinct npAAs can be achieved only when several proteinogenic amino acids are excluded from *in vitro* translation systems to create vacant codons that are assignable for npAAs, which results in the decreased building block repertoire. (2) Even when such an engineered translation system is used, it is impossible to efficiently synthesize polypeptides containing many kinds of *N*-methyl amino acids (^{Me}AAs) due to an unknown mechanism of translational disorder. In this context, there is a need to develop *in vitro* translation systems enabling (1) expansion of the building block repertoire by utilizing multiple npAAs in addition to the 20 proteinogenic ones and (2) the synthesis of nonstandard peptides containing a variety of ^{Me}AAs. As the Ph.D. degree research, I studied to develop these two kinds of engineered translation systems.

Expanding the amino acid repertoire of ribosomal polypeptide synthesis via artificial division of codon boxes

The genetic code contains eight family codon boxes consisting of synonymous codons that redundantly code for the same amino acid (Fig. 1A). For example, the four codons in the valine codon box (GUN; N=U, C, A, or G) code for valine by means of two specific tRNAs possessing GAC and cmo^{5}UAC (cmo^{5}U: uridine 5-oxyacetic acid) anticodons (Fig. 1A). If we could manipulate the decoding system and reduce the redundancy, more npAAs could be utilized without the need to sacrifice any of the 20 proteinogenic amino acids. To achieve this goal, we reconstituted an *in vitro* translation system with 32 *in vitro* transcribed tRNAs possessing SNN (S = G or C) anticodons (Fig. 1B). These 32 tRNA transcripts can be charged with 20 proteinogenic amino acids by endogenous aminoacyl-tRNA synthetases (AARSs) *in situ* and orthogonally decode the corresponding 31 NNS elongation codons as well as the AUG initiation codon (Fig. 1B). Among the 32 tRNAs, we can omit some redundant tRNAs and replace them with tRNA$^{AsnE2}_{SNN}$'s with appropriate anticodons, whose body sequence is engineered to avoid aminoacylation by endogenous AARSs. When these tRNA$^{AsnE2}_{SNN}$'s are precharged with different npAAs by artificial aminoacylation ribozymes 'flexizymes', the corresponding codons can be reassigned to npAAs without abandoning proteinogenic amino acids (Fig. 1C). By this strategy, I have artificially divided the GUN, CGN, and GGN codon boxes and reassigned three distinct npAAs to them, expanding the building block repertoire up to 23 (ref. 2,3).

The efficiency and accuracy of this novel translation system were improved by two kinds of contrivances. First, the problem of inefficient aminoacylation reaction for several tRNA transcripts, which lacked the posttranscriptional nucleoside modifications, was overcome by optimizing the individual concentrations of 32 tRNA transcripts. Second, the problem of translation inaccuracy caused by the artificial

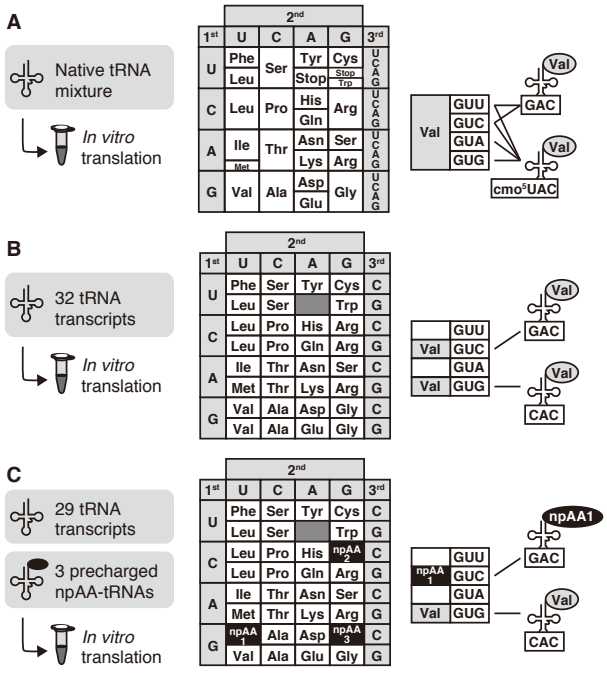

Fig. 1 Schematic representation of artificial division of codon boxes. (A) The genetic code of a reconstituted translation system containing *E. coli* native tRNA mixture. (B) A reprogrammed genetic code where 32 tRNA transcripts decode 31 NNS (S = C or G) elongation codons along with the AUG initiation codon. (C) A reprogrammed genetic code containing 23 building blocks by means of artificial division of three codon boxes.

codon-box division was diminished by optimizing several translation conditions including the concentrations of Mg^{2+} and npAA-tRNA^{AsnE2}, and reaction time. Using the optimal condition, I proofed the concept by expressing a 32-mer linear peptide consisting of 20 proteinogenic and 3 nonproteinogenic amino acids as well as a 14-mer macrocyclic peptide "CM_{11}-1" containing three kinds of ^{Me}AAs along with D-tryptophan (Fig. 2). In both cases, the correct peptide products were synthesized accurately, which demonstrated the successful expansion of the building block repertoire.

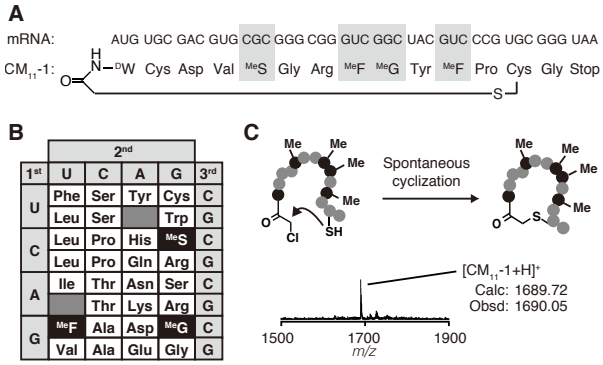

Fig. 2 Artificial division of three codon boxes to express an *N*-methylated macrocyclic peptide. (A) Sequences of mRNA and the corresponding peptide. ^{D}W: D-tryptophan, ^{Me}S: *N*-methylserine, ^{Me}F: *N*-methylphenylalanine, ^{Me}G: *N*-methylglycine. (B) A reprogrammed genetic code containing three ^{Me}AAs in artificially divided codon boxes. (C) MALDI-TOF-MS of the peptide product. The macrocyclic structure was formed by spontaneous cyclization reaction between chloroacetyl-^{D}W and the downstream Cys residue at the 13th position.

Ribosomal synthesis of highly *N*-methylated peptides

In terms of nonstandard peptide drug development, ^{Me}AAs are one of the most attractive npAAs since the *N*-methyl modification can improve the peptide's cell membrane permeability and stability against enzymatic decomposition. However, the synthetic efficiency of *N*-methylated peptides is often less than the case of peptides with side chain modification, thus it is difficult to utilize multiple distinct ^{Me}AAs simultaneously. The best case in literature so far is the synthesis of a peptide containing only three kinds of ^{Me}AAs. Regarding this problem, I hypothesized the mechanism of the inefficiency as follows. Ribosome recruits aminoacyl-tRNAs by means of EF-Tu protein (elongation factor thermo unstable) and accommodates them into the A-site space (Fig. 3). EF-Tu has similar binding affinities to 20 kinds of proteinogenic aminoacyl-tRNAs, and therefore it can accommodate all of them with similar efficiencies. In contrast, if the affinity between EF-Tu and an *N*-methyl-aminoacyl-tRNA (^{Me}AA-tRNA) were weak, the ^{Me}AA-tRNA could not bind to EF-Tu under the competition with proteinogenic aminoacyl-tRNAs, which would lead to decrease in the synthetic efficiency of the ^{Me}AA-containing peptide.

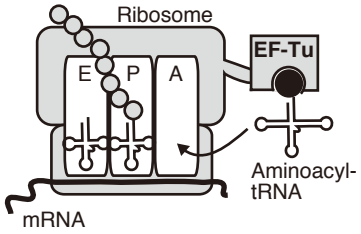

Fig. 3 Scheme of EF-Tu-mediated accommodation of an aminoacyl-tRNA into ribosome.

Based on this hypothesis, I studied to change the affinity between each ^{Me}AA-tRNA^{AsnE2} and EF-Tu. (Details are omitted due to the reasons involving the patent application and future publication). The effect of affinity change was examined by the ribosomal synthesis of a model peptide containing multiple distinct ^{Me}AAs. When the translation was conducted by the conventional system, the products were a mixture of the desired peptide and various side products containing proteinogenic amino acids in place of ^{Me}AAs. On the other hand, the optimized system developed in this study yielded only the desired peptide with improved expression level. The results demonstrated that the cause of translational disorder can be attributed to the insufficient EF-Tu affinities of ^{Me}AA-tRNAs, and also the study provided a practical methodology to synthesize highly *N*-methylated peptides containing a variety of ^{Me}AAs.

Conclusion

In this study, I have developed the engineered translation systems that allow for the synthesis of nonstandard peptides that comprise expanded repertoire of proteinogenic and nonproteinogenic amino acids. These technologies will open a new opportunity for the synthesis of polypeptides containing a rich variety of npAAs. In particular, the integration of these engineered translation systems with peptide selection technologies, such as the RaPID system, would enable us to express a library of nonstandard macrocyclic peptides with highly modified scaffolds and to discover nonstandard peptide drugs that possess improved binding potencies and pharmacokinetic properties in future.

References

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