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Adding L-lysine derivatives to the genetic code of mammalian cells with engineered pyrrolysyl-tRNA synthetases

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ABSTRACT

We report a method for site-specifically incorporating L-lysine derivatives into proteins in mammalian cells, based on the expression of the pyrrolysyl-tRNA synthetase (PylRS)-tRNA^{Pyl} pair from *Methanosarcina mazei*. Different types of external promoters were tested for the expression of tRNA^{Pyl} in Chinese hamster ovary cells. When tRNA^{Pyl} was expressed from a gene cluster under the control of the U6 promoter, the wild-type PylRS-tRNA^{Pyl} pair facilitated the most efficient incorporation of a pyrrolysine analog, N^{ε}-*tert*-butyloxycarbonyl-L-lysine (Boc-lysine), into proteins at the amber position. This PylRS-tRNA^{Pyl} system yielded the Boc-lysine-containing protein in an amount accounting for 1% of the total protein in human embryonic kidney (HEK) 293 cells. We also created a PylRS variant specific to N^{ε}-benzyloxycarbonyl-L-lysine, to incorporate this long, bulky, non-natural lysine derivative into proteins in HEK293. The recently reported variant specific to N^{ε}-acetyllysine was also expressed, resulting in the genetic encoding of this naturally-occurring lysine modification in mammalian cells.

The biosynthesis of proteins containing non-natural amino acids, or alloproteins [1], is a promising way to facilitate protein science and molecular biology. Non-natural chemical groups, such as photo-inducible crosslinkers and fluorescent groups, and naturally-occurring post-translational modifications have thus been incorporated into proteins [2–4]. The cell-based, site-specific incorporation of non-natural amino acids into proteins relies on the expression of the pair of a tRNA and an aminoacyl-tRNA synthetase (aaRS) variant specific to a non-natural amino acid in the host organism [2]. This aaRS-tRNA pair should not cross-react with any endogenous aaRS or tRNA species in the host cell, thus being "orthogonal" to the host aminoacylation system [5,6]. Therefore, an archaeal or eukaryal aaRS-tRNA pair has been used in *Escherichia coli* [2,7,8], whereas a bacterial pair has been employed in *Saccharomyces cerevisiae* and mammalian cells [2–4].

The employed tRNA species are engineered to recognize nonsense codons, the amber (UAG), ochre (UAA), and opal (UGA) codons, and four-letter codons [2,9,10]. On the other hand, a variety of aaRS variants that specifically recognize non-natural amino acids have been created by engineering tyrosyl-tRNA synthetases from *E. coli* [2–4] and *Methanocaldococcus jannaschii* [2]. Tryptophanyl-tRNA synthetase [2], phenylalanyl-tRNA synthetase [7,8], lysyl-tRNA synthetase [2], and leucyl-tRNA synthetase [2] have also produced useful variants. Thus, the genetic code has been expanded to include a variety of amino acids with an aromatic ring bound to the β-methylene group and several other amino acids with different backbone structures.

Pyrrolysyl-tRNA synthetase (PylRS), found in certain archaeal and bacterial species, directly esterifies tRNA^{Pyl} with pyrrolysine (Fig. 1), a lysine derivative with the N^{ε} atom in an amide linkage with a pyrroline ring [11,12]. tRNA^{Pyl} is a natural amber suppressor, and it has non-canonical primary and secondary structures that are recognized by PylRS. The introduction of the archaeal PylRS-tRNA^{Pyl} pair into *E. coli* cells reportedly resulted in the incorporation of pyrrolysine and its analog, N^{ε}-cyclopentyloxycarbonyl-L-lysine (Cyc), into proteins at the amber position [12–14]. The engineering of PylRS could facilitate the incorporation of non-natural amino acids with a bulky chemical group at the N^{ε} atom of L-lysine. The structural basis for the substrate recognition has been elucidated by determining the crystal structures of *Methanosarcina mazei* PylRS complexed with its amino acid substrates [15–17]. The pyrroline ring of pyrrolysine as well as the pentane ring of Cyc is buried in a large, hydrophobic cavity at one end of the deep amino-acid-binding pocket of the enzyme. Based on the crystal structure, a variant specific to N^{ε}-acetyllysine (AcLysRS) was recently created from *Methanosarcina barkeri* PylRS for use in *E. coli* cells;

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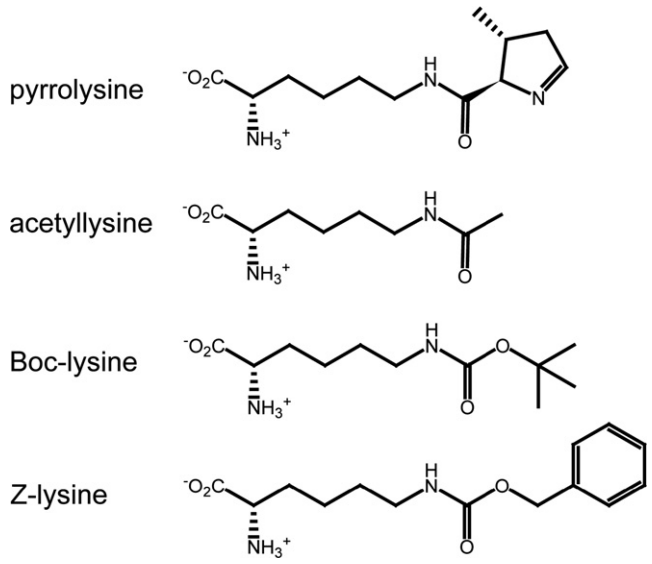

Fig. 1. Chemical structures of the L-lysine derivatives.

the large, hydrophobic cavity is plugged in this variant by substitutions with bulky amino acids [18].

In the present study, we developed a method for the efficient, site-specific incorporation of L-lysine derivatives in mammalian cells with the PylRS-tRNA^{Pyl} pair from *M. mazei*. This method was applied, together with engineered PylRS variants, to incorporate N^{ε}-benzyloxycarbonyl-L-lysine (Z-lysine) (Fig. 1), which is larger than pyrrolysine, and acetyllysine into proteins.

Materials and methods

Amino acids. Boc-lysine, Z-lysine, and acetyllysine were purchased from Bachem. Z-lysine was dissolved in 1 M HCl at a concentration of 100 mM. Growth media supplemented with this solution were neutralized with 1 N NaOH before use.

Plasmids used in mammalian cells. Four tRNA^{Pyl} genes were constructed. The first gene consisted of the tRNA^{Pyl} coding sequence (GGAAACCTGATCATGTAGATCGAATGGACTCTAAATCCGTTCAGCCG GGTTAGATTCCCGGGGTTTCCG), followed by the terminator (GACA AGTGCGGTTTTT), being cloned in the vector pCR4 Blunt-TOPO (Invitrogen). The second consisted of the 5′-leader of the human tRNA^{Tyr} gene (AGCGCTCCGGTTTTTCTGTGCTGAACCTCAGGGGACGC CGACACACGTACACGTC), the human tRNA^{Val} sequence (GTTTCCG TAGTGTAGTGGTCATCACGTTCGCCTAACACGCGAAAGGTCCCCGGTT CGAAACCGGGCGGAAACA), a spacer (AGATCTTCTAGACTCGAG), the tRNA^{Pyl} sequence, and the terminator in this order, being cloned in pCR4 Blunt-TOPO. The third consisted of the human U6 promoter with the CMV enhancer [19], the spacer, tRNA^{Pyl}, and the terminator in this order, being cloned in pUC119. The fourth consisted of the T7 promoter, the spacer, tRNA^{Pyl}, and the terminator in this order, being cloned in pBR322. Nine tandem copies of the U6-tRNA^{Pyl} gene lacking the CMV enhancer were cloned in the SspI-EcoRV sites of pBR322. The wild-type PylRS gene, with an N-terminal FLAG tag, was cloned in the vector pcDNA3.1/Zeo(+) (Invitrogen). The T7 RNA polymerase gene was cloned in pcDNA4/TO to create pT7RNAP. The LacZ(Am91) and GRB2(Am111) genes were previously described [20]. Twenty-four residues (MASMTGGQQMGRD PGANSGVTKNS) were added at the N-terminus of the glutathione *S*-transferase (GST) gene from the vector pGEX (Amersham Biosciences). Met at the original N-terminus, now at position 25, was mutated into the amber codon to create GST(Am25). The oriP sequence from pCEP4 (Invitrogen) was cloned in the MunI site of the pcDNA4/TO lacking the sequence between the two PvuII sites, to create pOriP. For experiments with HEK293 c-18, the PylRS, LacZ(Am91) and GST(Am25) genes and 9×tRNA^{Pyl}, except for GRB2, were carried on pOriP.

Amber suppression in mammalian cells. The plasmid transfection and the analysis of the expression of reporter genes were performed as described previously [3,4,20]. HEK293 c-18 cells were from ATCC (No.CRL-10852). To express tRNA^{Pyl}, PylRSs, and reporter genes, CHO or HEK293 c-18 cells were transfected with the corresponding expression plasmids. As for the expression of the T7-tRNA^{Pyl} gene, CHO cells were transfected with the PCR product of this gene and pT7RNAP. After transfection, the cells were incubated in the presence/absence of 1 mM Boc-lysine/Z-lysine in growth media for 16 h (CHO) and for 40 h (HEK293) before analysis. For the incorporation of acetyllysine, both types of cells were incubated for 16 h after transfection.

Amber suppression in E. coli cells. The plasmids used for the expression of tRNA^{Pyl} and PylRS in *E. coli* are described, together with the methods for PylRS engineering, in Supplementary material.

Results

Site-specific incorporation of Boc-lysine into proteins in CHO cells with the wild-type PylRS and tRNA^{Pyl} pair

A pyrrolysine analog, N^{ε}-*tert*-butyloxycarbonyl-L-lysine (Boc-lysine), is recognized by the wild-type PylRS (Yanagisawa et al., unpublished data). This pair of Boc-lysine and the wild-type PylRS was used to develop a tRNA^{Pyl} expression system suitable for the efficient incorporation of L-lysine derivatives into proteins in mammalian cells. Amber mutant genes for GRB2 and LacZ were used as reporter genes to analyze the amber suppression caused by the PylRS-tRNA^{Pyl} pair, together with Boc-lysine supplemented in growth media at a concentration of 1 mM. The GRB2 and LacZ genes contained an amber codon at positions 111 and 91, respectively. The GRB2 gene was tagged with a C-terminal FLAG. The full-length product of GRB2(Am111)-FLAG is larger than that of the endogenous GRB2. An anti-GRB2 antibody detected both of these GRB2 molecules.

Most of the prokaryotic tRNA genes, including tRNA^{Pyl}, lack the internal promoter sequence found among the eukaryotic tRNA genes, and are not expected to be expressed in eukaryotic cells. In fact, the introduction of the coding sequence for tRNA^{Pyl} (Fig. 2A, P1), together with the wild-type PylRS gene, into Chinese hamster ovary (CHO) cells was not sufficient for the expression of the GRB2 and LacZ reporter genes (Fig. 2B and C, respectively). Therefore, we examined the applicability of external promoters [21–24] for the expression of tRNA^{Pyl}. The human tRNA^{Val} gene lacking its transcriptional terminator, the U6 promoter, and the bacteriophage T7 promoter were linked to the 5′ end of the coding sequence for tRNA^{Pyl} (Fig. 2A). The U6 promoter was used together with the CMV enhancer to increase the amount of transcript [19], and for the use of T7 promoter, T7 RNA polymerase was expressed in the cell. Amber suppression occurred to produce GRB2-FLAG, when tRNA^{Pyl} was expressed using the human tRNA^{Val} sequence, the U6 promoter, and the T7 promoter (Fig. 2B, lanes 9, 13, and 17, respectively) each in the presence of PylRS and Boc-lysine. The expressed GRB2-FLAG probably contained Boc-lysine at the amber position, because the amber suppression did not occur when Boc-lysine was removed from the growth medium (Fig. 2B, lanes 8, 12, and 16).

Among the tested promoters, the U6 promoter provided the highest yield of GRB2-FLAG, which was comparable to the amount of endogenous GRB2 (Fig. 2B, lane 13). The β-galactosidase activity