Supplementary Material (ESI) for Molecular BioSystems

Improving Orthogonal tRNA-Synthetase Recognition for Efficient Unnatural Amino Acid Incorporation and Application in Mammalian Cells

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SUPPLEMENTARY METHODS

Materials

For DNA preparation and cloning, DH10B *E. coli* cells were used. Polymerase chain reaction (PCR) was carried out with Phusion™ high-fidelity DNA polymerase (New England Biolabs). Bzo, Azi, and Ome was purchased from Chem-Impex. Pyo was synthesized as described.[1] Other chemicals used in experiments were purchased from Sigma-Aldrich.

Construction of plasmids

All plasmids were cloned using standard methods and was confirmed by DNA sequencing.

The plasmid pSNR-TyrRS expressing *E. coli* $tRNA^{Tyr}_{CUA}$ and wild-type TyrRS was used in yeast experiments.[2] Mutations were placed in the synthetase by digesting the plasmid with *BamH* I and *Xho* I. *BamH* I is located in the synthetase upstream from Asp265 and *Xho* I is located at the end of the gene. The region containing the mutation was amplified from pSNR-TyrRS using primers JT338 5'-CCA GTT CTG GAT CAA CAC TGC GCA AGC CGA CGT TTA CCG CTT CC-3' (for Asp265Gln), JT339 5'-CCA GTT CTG GAT CAA CAC TGC GTT CGC CGA CGT TTA CCG CTT CC-3' (for Asp265Phe), JT340 5'-CCA GTT CTG GAT CAA CAC TGC GTT GGC CGA CGT TTA CCG CTT CC-3' (for Asp265Leu), JT341 5'-CCA GTT CTG GAT CAA CAC TGC GTA CGC CGA CGT TTA CCG CTT CC-3' (for Asp265Tyr), and JT342 5'-CCA GTT CTG GAT CAA CAC TGC GAG AGC CGA CGT TTA CCG CTT CC-3' (for Asp265Arg) with primer FW22 5'-AAC TCG AGT TAT TTC CAG CAA ATC AGA CAG-3'. The products were re-amplified with primer JT313 5'-GTT GGA TCC GAA GAA AAC CAG CCC GTA CAA ATT CTA CCA GTT CTG GAT CAA CAC TGC G-3' and FW22 to append the sequence of the synthetase to the *BamH* I site. Amplified sequences were digested with *BamH* I and *Xho* I and inserted at the *BamH* I and *Xho* I site of pSNR-TyrRS.

The BzoRS, AziRS, OmeRS, and PyoRS genes were all constructed from *E. coli* TyrRS gene through site-directed mutagenesis. They contained the following mutations: BzoRS (Y37G, D182G, and L186A); AziRS (Y37L, D182S, F183A and L186A); OmeRS (Y37T, D182T, and L183M); and PyoRS (Y37G, D182S, and F183M). To incorporate unnatural amino acids in mammalian cells, the expression plasmid pEYcua-TyrRS was digested with *BamH* I and *Nhe* I.[3] The mutated synthetase genes were amplified using primer JT313 and EYRS-C 5'-ACA AGA TCT GCT AGC TTA TTT CCA GCA AAT CAG ACA GTA ATT C-3'. The PCR products were digested with *BamH* I and *Nhe* I and inserted into the precut pEYcua-TyrRS to afford pEYcua-BzoRS, pEYcua-AziRS, pEYcua-OmeRS, and pEYcua-PyoRS.

The Asp265Arg mutation was cloned into BzoRS, AziRS, OmeRS, and PyoRS. Using pSNR-ETyrRS containing the TyrRS(Asp265Arg) as a template, the region between the internal *BamH* I site and the *Nhe* I site was amplified using primers JT313 and EYRS-C. The amplified fragment was digested with *BamH* I and *Nhe* I and ligated into the precut pEYcua-BzoRS, pEYcua-AziRS, pEYcua-OmeRS, and pEYcua-PyoRS to make the enhanced version of each synthetase pEYcua-EBzoRS, pEYcua-EAziRS, pEYcua-EOmeRS, and pEYcua-EPyoRS for use in mammalian cells.

Plasmid used for mammalian expression of Glutathion S-transferase (GST) contained a Cytomegolovirus (CMV) promoter and BGH polyA. GST was amplified from *E. coli* genomic DNA using primer GSTfor 5'-TAG TCG GTA CCA TGA AAT TGT TCT ACA AAC CGG GTG CCT GC -3' and primer GST6Hisrev 5'- AGT CCT CGA GTT AGT GGT GGT GGT GGT GGT GCT TTA AGC CTT CCG CTG AC-3'. GST mutants were amplified using overlapping PCR. Internal primers for GST N87TAG were GST87tagFor 5'-CAG TTG CTG GCA CCG GTA TAG AGT ATT TCC CGC TAT AAA ACC ATC-3' and GST87tagRev 5'-TTT ATA GCG GGA AAT ACT CTA TAC CGG TGC CAG CAA CTG GCG GTC-3', for GST Y92TAG were GST92tagFor 5'- GTA AAC AGT ATT TCC CGC TAG AAA ACC ATC GAA TGG CTG AAT TAC-3'and GST92tagRev 3'-TCA GCC ATT CGA TGG TTT TCT AGC GGG AAA TAC TGT TTA CCG GTG-3', and for GST V125TAG were GST125tagFor 5'-GAA GAG TAC AAA CCG ACA TAG CGC GCG CAG CTG GAG AAG AAG CTG-3' and GST125tagRev 5'-TCT TCT CCA GCT GCG CGC GCT ATG TCG GTT TGT ACT CTT CCG GTG-3'. FLAG-tag GST and GST mutants were amplified from PCR products using primer JT375 5'-GAG GCT AGC GCC ACC ATG AAA TTG TTC TAC AAA CCG GGT GCC-3' and JT376 5'-GAG GGA TCC TTA TTT ATC ATC ATC ATC TTT GTA ATC CTT TAA GCC TTC CGC TGA CAG CG-3'. Amplified products were digested with *Kpn* I and *Xho* I and ligated into precut pCDNA3 (Invitrogen).

Cell culture and transfection

A clonal HeLa GFP-TAG reporter stable cell line[3] and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% fetal bovine serum (FBS, Mediatech). Cells were transfected with plasmid DNA using Lipofectamine 2000 according to the protocol of the vendor (Invitrogen).

Flow cytometry

Hela-GFPtag cells seeded in a 3.5 cm culture dish were transfected with 5 μg of pEYcua-aaRS plasmid DNA using Lipofectamine 2000. Hela-GFPtag cells that were not transfected with a tRNA/synthetase pair were used as a negative control. UAA was added 24 h post transfection and FACS analysis was carried out 24 h after the addition of the UAA. Cells were trypsinized and washed with PBS twice. Samples were centrifuged and re-suspended in 1 mL PBS and 10 μL of propidium iodide. Samples were analyzed with a FACScan (Becton & Dickinson). The excitation wavelength was 488 nm, and the emission filter was 530/30 nm. For each sample the total fluorescence intensity of 30,000 cells was recorded, and was normalized to the total fluorescence intensity of cells transfected with pEYcua-TyrRS.

Photocrosslinking and Western blot analysis

293T cells seeded in a 3.5 cm culture dish were transfected with pCDNA-GST-His (TAG mutants) and pEYcua-EAziRS or pEYcua-ETyrRS. Unnatural amino acid Azi was added to the growth media of the appropriate samples in the final concentration of 1 mM. After 48 hours, media was removed and replaced with phosphate buffer saline (PBS). The sample was kept on ice, and a 365 nm UV lamp was placed 1 cm above the sample. Samples were exposed under UV light for 10 minutes. Cells were removed by pipette, lysed on ice with 30 μL of 1% Nonidet P-40 (NP40, Calbiochem) in PBS. After centrifugation, 5 μL of supernatant from cells transfected with pEYcua-ETyrRS and 15 μL of pEYcua-EAziYRS supernatant were loaded and separated by 12% SDS-PAGE. An antibody (Penta-His HRP conjugate Kit, Qiagen) against the His6 tag was used to detect GST proteins before and after photocrosslinking.

For FLAG-tag westerns, 293T cells were seeded in a 10 cm dish for transfection with pCDNA-GST-FLAG (TAG mutants) and pEYcua-TyrRS, pEYcua-ETyrRS, pEYcua-AziRS or pEYcua-EAziRS. Crosslinking was carried out under UV light for 15 minutes. Cells were removed by 1 mL cold PBS and lysed by 3 rounds of liquid nitrogen to 37°C water bath freeze-thaw. After centrifugation, the supernatant was loaded onto 15 μL packed volume of glutathione sepharose 4B (GE Healthcare) and GST was purified according to the protocol of the vendor. After purification GST proteins were eluted and boiled in SDS loading buffer. Five microliters from pEYcua-TyrRS or pEYcua-ETyrRS transfected samples and 15 μL from pEYcua-AziYRS or pEYcua-EAziYRS transfected samples were loaded and separated by 12% SDS-PAGE. The primary antibody (Monoclonal ANTI-FLAG M2, Sigma-Aldrich) against the FLAG tag and HRP conjugated secondary antibody (Goat Anti-Mouse IgG (H+L)-HRP Conjugate, Bio-Rad) was used to detect GST proteins.

References

1. A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson and P. G. Schultz, *J. Am. Chem. Soc.* 2003, **125**, 11782-11783.
2. Q. Wang and L. Wang, *J. Am. Chem. Soc.* 2008, **130**, 6066-6067.
3. W. Wang, J. K. Takimoto, G. V. Louie, T. J. Baiga, J. P. Noel, K. F. Lee, P. A. Slesinger and L. Wang, *Nat. Neurosci.*, 2007, **10**, 1063-1072.