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LETTER

A Genetically Encoded Metabolically Stable Analogue of Phosphotyrosine in *Escherichia coli*

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ABSTRACT *p*-Carboxymethyl-L-phenylalanine (*p*CMF), a phosphotyrosine (pTyr) mimetic that is resistant to protein tyrosine phosphatase hydrolysis, was cotranslationally incorporated into proteins in *Escherichia coli* using an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase (aaRS) pair. The *p*CMF-specific aaRS was identified from a large library of *Methanococcus jannaschii* tyrosyl-tRNA synthetase active-site mutants by a combination of positive and negative genetic selections. When *p*CMF was substituted for Tyr701 in human signal transducer and activator of transcription-1 (STAT1), a constitutively active mutant was obtained that dimerizes and binds a DNA oligonucleotide duplex that contains the M67 site recognized by Tyr701-phosphorylated STAT1. Genetic incorporation of *p*CMF into proteins should provide a new tool for the preparation of stable analogues of a wide array of phosphoproteins involved in signal transduction pathways, as well as the development of peptide-based, cellularly expressed inhibitors of pTyr binding proteins.

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Protein phosphorylation, principally on serine, threonine, or tyrosine residues, plays a critical role in virtually all cellular signal transduction pathways. The dynamic interconversion of phosphorylated and dephosphorylated isoforms of a protein (*1, 2*), however, can complicate both biochemical and cellular studies of the roles of individual phosphorylation sites. Consequently, the replacement of phosphotyrosine (pTyr) in phosphoproteins with an analogue that retains functional activity but is resistant to hydrolysis by protein tyrosine phosphatases (PTPs) would facilitate functional studies of protein phosphorylation. One broadly used, stable analogue of pTyr is 4-phosphonomethyl-L-phenylalanine (Pmp), in which the phosphoryl ester oxygen atom is replaced by a methylene group (*3*) (Figure 1, panel a). For example, Cole and coworkers used expressed protein ligation to substitute Pmp for pTyr residues in the pTyr phosphatases SHP-1 (*4*) and SHP-2 (*5, 6*) to determine the function of individual phosphorylation sites. This method, which relies on solid-phase peptide synthesis to introduce Pmp into the peptide fragment for subsequent ligation, is principally limited to amino acid substitutions at the N- or C-terminal regions of proteins (*7, 8*). Recently, we developed methodology that allows the incorporation of unnatural amino acids into proteins at sites specified by nonsense or frameshift codons in *Escherichia coli*, yeast, and mammalian cells (*9*–*13*). Here we report the generation and use of an orthogonal amber-suppressor transfer RNA (tRNA)/aminoacyl-tRNA synthetase (aaRS) pair that allows the selective incorporation of the non-hydrolyzable pTyr mimetic, *p*-carboxymethyl-L-phenylalanine (*p*CMF, **3**; Figure 1, panel a), at any desired site in proteins in *E. coli*.

We initially attempted to genetically encode Pmp in *E. coli*, but unfortunately this unnatural amino acid has poor bioavailability, which results in very low cytoplasmic concentrations of Pmp and complicates its *in vivo* incorporation into proteins. However, studies of cell-permeable peptide inhibitors of SH2 domains found that a group of carboxy-based analogues with improved cellular permeability (*14*) could be functionally substituted for pTyr. For example, *p*CMF **3** (Figure 1, panel a) was used to mimic pTyr in a pentapeptide-based inhibitor of the interaction between the Src SH3–SH2 domain and phosphorylated epidermal growth factor receptor, albeit with a significant decrease in potency (*15*). Similar results were seen in the design of a cell-permeable inhibitor to the growth factor receptor-bound 2 (Grb2) SH2 domain (*16*). Molecular modeling (*16*) and X-ray crystallographic analyses (*17*) show that the side chain of *p*CMF ($-CH_2COO^-$) has good spatial overlap with two of the pTyr phosphate oxygen atoms that interact with positively charged arginine residues in SH2 domains (*18, 19*). In addition, carboxylic acids have

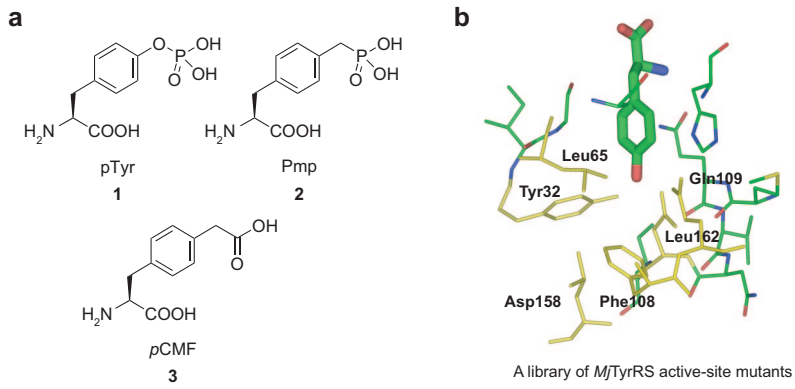

Figure 1. Evolution of *Mj*TyrRS variants for the selective incorporation of *p*CMF into proteins. a) Structure of pTyr, Pmp, and *p*CMF. b) A library of *Mj*TyrRS active-site mutants was generated by randomizing six residues (in yellow) in the tyrosine binding site. Residues for randomization were selected on the basis of observed contacts between *Mj*TyrRS and tyrosine in the crystal structure of *Mj*TyrRS–tRNA^{Tyr}–L-tyrosine complex (*30*).

also been used to mimic other phosphorylated amino acids; for example, glutamic acid was substituted for Ser389 in the tumor suppressor protein p53 to afford an effective mimic of activated, phosphorylated p53 (*20*). We therefore focused our efforts on the genetic incorporation of *p*CMF into proteins in *E. coli*.

*p*CMF was synthesized in four steps from 2-((4-bromomethyl)phenyl)acetic acid (see Supporting Information). Its cellular bioavailability was then tested by a method (*21, 22*) in which *E. coli* DH10B cells were grown to saturation in minimal media supplemented with 1.0 mM *p*CMF, and the harvested cells were washed, lysed, and analyzed by LC/MS. A signal corresponding to ≥100 μM of *p*CMF in the cytoplasm was found; for comparison, the cellular concentrations of tyrosine, tryptophan, and phenylalanine were 800, 200, and 300 μM, respectively (*21*). Previous experiments have shown that D-amino acids are not incorporated by the ribosome; therefore the racemic amino acid was used in these studies (*21–29*).

Next, an orthogonal tRNA/aaRS pair was evolved that selectively incorporates *p*CMF into proteins in *E. coli*. This pair was generated from an *M. jannaschii* amber-suppressor tyrosyl tRNA(*Mj*tRNA$^{Tyr}_{CUA}$)/tyrosyl-tRNA synthetase (*Mj*TyrRS) pair by a directed evolution strategy previously used for the incorporation of a variety of other unnatural amino acids in *E. coli* (*9, 22*). A library of *Mj*TyrRS active-site mutants encoded in plasmid pBK-lib-2 was generated in which six residues (Tyr32, Leu65, Phe108, Gln109, Asp158, and Leu162) within 6.5 Å of the tyrosine substrate were randomized based on the structure of the *Mj*TyrRS–tRNA^{Tyr}–L-tyrosine complex (Figure 1, panel b) (*30*). The library was constructed by overlap extension polymerase chain reaction (*31*) using synthetic degenerate oligonucleotide primers to introduce mutations, where the intended mutation residues were encoded by the degenerate codon NNK (N = A+T+C+G, K = T+G).

This library was then passed through rounds of alternating positive and negative selections (*9, 22*). The positive selection is based on resistance to chloramphenicol, which is conferred by suppression of an amber mutation at a permissive site in the chloramphenicol acetyl transferase gene in the presence of 1 mM unnatural amino acid and *Mj*TyrRS (*9*). The negative selection utilizes the toxic barnase gene with amber mutations at permissive sites and is carried out in the absence of the unnatural amino acid. Cells containing *Mj*TyrRS variants that acylate the orthogonal *Mj*tRNA$^{Tyr}_{CUA}$ with *p*CMF survive the positive selection, whereas cells containing *Mj*TyrRS variants that acylate *Mj*tRNA$^{Tyr}_{CUA}$ with endogenous amino acids express barnase and die. After five rounds of selection (positive, negative, positive, negative, and positive), 26 clones were isolated that survive on 120 μg mL^{-1} chloramphenicol in the presence of 1 mM *p*CMF but die on 20 μg mL^{-1} chloramphenicol in the absence of *p*CMF. DNA sequencing revealed five unique mutants (Table 1) with *p*CMFRS#1 and *p*CMFRS#2 each occurring 11 times. In most of the mutants, Tyr32 and Asp158 were both mutated to smaller residues, which should expand the active site to accommodate the larger side chain of *p*CMF. These mutations also remove the hydrogen bonds in the wild-type enzyme to the phenolic hydroxy group of bound tyrosine, lead-

TABLE 1. The 26 selected *p*CMF-specific *Mj*TyrRS mutants represent five unique mutants[a]

WT *Mj*TyrRS	Frequency	Tyr32	Leu65	Phe108	Gln109	Asp158	Leu162
*p*CMFRS#1	11 clones	Ser	Ala	Lys	His	Gly	Lys
*p*CMFRS#2	11 clones	Ser	Ala	Ala	Gln	Gly	Lys
*p*CMFRS#3	2 clones	Ala	Ser	Arg	Asn	Ser	His
*p*CMFRS#4	1 clone	Ser	Ala	Asn	Tyr	Gly	Lys
*p*CMFRS#5	1 clone	Ala	Arg	Gln	Gly	Lys	Ala

[a]Both the mutant sequences and the frequencies of each *Mj*TyrRS mutant are given.

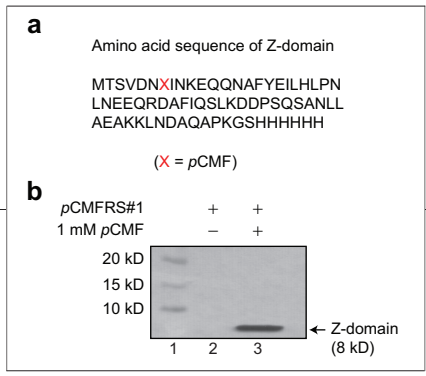

Figure 2. Verification of the fidelity and efficiency of *p*CMF incorporation into proteins in response to the amber codon by the evolved *p*CMFRS#1/*Mj*tRNA$^{Tyr}_{CUA}$ pair. a) Amino acid sequence of the mutant Z-domain protein whose gene contains a Lys7 → TAG mutation. b) Analysis of the mutant Z-domain protein by SDS-PAGE and silver staining. Proteins were purified by Ni^{2+} affinity chromatography.

ing to a loss of affinity for the natural substrate. In addition, Phe108 and Leu162 were mutated to basic amino acids at a high frequency in the selected mutants; these residues likely form a salt bridge with the carboxylate moiety of *p*CMF.

The fidelity and efficiency of *p*CMF incorporation into proteins by the *p*CMFRS#1/*Mj*tRNA$^{Tyr}_{CUA}$ pair was further verified by expressing a Z-domain protein (with a C-terminal His_6 tag), whose gene contains a Lys7 → TAG mutation (Figure 2, panel a). When the *p*CMFRS#1/*Mj*tRNA$^{Tyr}_{CUA}$ pair is used to suppress the amber codon in the presence of 1 mM *p*CMF, full-length Z-domain protein is produced as indicated by SDS-PAGE analysis (Figure 2, panel b, lane 3). The yield of mutant protein was 1.2 mg from 1 L of minimal media. In the absence of *p*CMF, no Z-domain protein was detectable by silver staining (Figure 2, panel b, lane 2), indicating that *p*CMF is incorporated only in response to the amber codon. MALDI–TOFMS analysis of the purified Z-domain mutant gave two peaks (see Supporting Information), corresponding to the intact mutant Z-domain protein ($M_{experimental}$ = 7966 Da, $M_{theoretical}$ = 7966 Da) and the mutant Z-domain protein without the first methionine moiety ($M_{experimental}$ = 7833 Da, $M_{theoretical}$ = 7834 Da). The *p*CMF-dependent expression of amber-mutant Z-domain protein and corresponding mass spectral analysis confirm a high fidelity for the incorporation of *p*CMF but not tyrosine or other endogenous amino acids by this method.

To test the ability of *p*CMF to mimic pTyr in a naturally phosphorylated protein, a fragment (132–712) of human signal transducer and activator of transcription-1 (STAT1) (which is expressed in *E. coli* in a stable form) was used as a model system (*32*). STAT1 has a DNA-binding domain (residues 317–488) and an internal SH2 domain (residues 576–683) (Figure 3, panel a). Phosphorylation of STAT1 on Tyr701 leads to homodimerization, and the resulting dimer binds tightly to DNA that contains M67 sites (*33*). To selectively substitute *p*CMF for pTyr701 in STAT1, the codon encoding Tyr701 was mutated to TAG and then suppressed with the evolved *p*CMFRS#1/*Mj*tRNA$^{Tyr}_{CUA}$ pair in the presence of 1 mM *p*CMF in *E. coli*. The Tyr701 *p*CMF mutant of STAT1 was expressed and purified to homogeneity by ammonium sulfate precipitation, anion-exchange chromatography, and size-exclusion chromatography to afford 2.9 mg of protein from 1 L of minimal media (Figure 3, panel b). A 21-bp DNA oligonucleotide duplex that contains an M67 site was then radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (PNK) (Figure 3, panel c) and used to determine the binding affinity of *p*CMF-containing STAT1. As indicated by an electrophoretic mobility shift assay (EMSA) on a native 4–20% Novex Tris–borate–EDTA (TBE) gel (Figure 3, panel d), the DNA duplex was bound by the Tyr701 *p*CMF mutant of STAT1 with a K_{app} of 21 nM. For comparison, the K_{app} of pTyr701 STAT1 is reported to be 1 nM (*34*), and the lower limit for detection of DNA complexes with wild-type STAT1 was with 125 nM protein (see Supporting Information). These results indicate that the Tyr701 *p*CMF mutant of

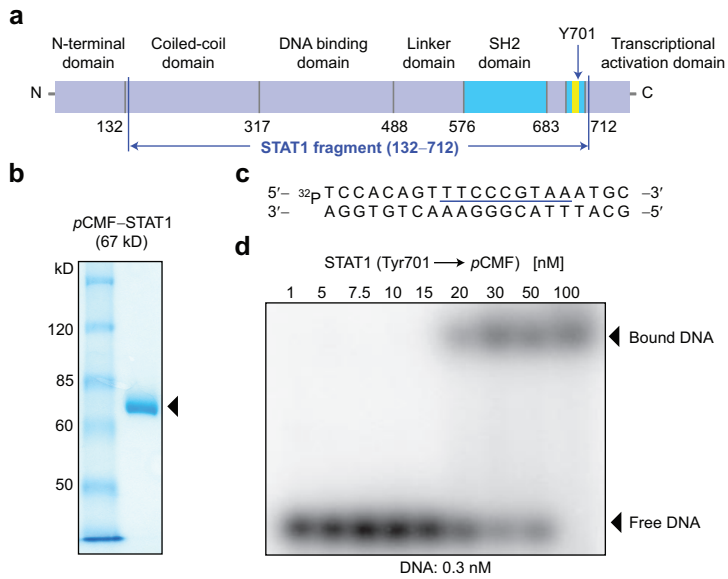

Figure 3. EMSAs show that the Tyr701 → *p*CMF mutant of STAT1 binds M67-containing DNA duplex. a) Schematic diagram showing the domains of human STAT1. b) SDS-PAGE analysis of the expressed and purified Tyr701 *p*CMF mutant of STAT1 fragment (132–712). The proteins were stained by GelCode blue staining reagent (Pierce). c) Sequence of the 21-bp DNA oligonucleotide duplex used in this study. The M67 site is underlined in the top strand. The DNA is labeled with ^{32}P by T4 PNK. d) EMSA showing that the Tyr701 *p*CMF mutant of STAT1 binds the M67-containing DNA duplex. The DNA concentration was fixed at 0.3 nM and titrated in a 12.5 μL volume against a standard protein dilution series. The products were resolved on a native 4–20% Novex TBE gel (Invitrogen).

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STAT1 dimerizes and binds the M67-containing DNA duplex in a similar fashion to Tyr701-phosphorylated STAT1. Thus *p*CMF can serve as a functional mimic of pTyr when incorporated site-specifically into proteins. However, the absolute affinity of binding interactions involving *p*CMF will likely depend on the specific context in which it is used.

In conclusion, an orthogonal *Mj*tRNA$^{Tyr}_{CUA}$/*Mj*TyrRS pair was evolved that allows the selective incorporation of *p*CMF, a mimetic of pTyr, into proteins in response to an amber codon in *E. coli* with high fidelity and efficiency. When *p*CMF was substituted for pTyr in STAT1, the resulting protein dimerized and bound DNA in a similar manner to Tyr701-phosphorylated STAT1 protein. Because *p*CMF is resistant to hydrolysis by PTPs, it should prove a useful tool for the generation of stable analogues of selectively phosphorylated proteins and peptides.

METHODS

Expression and Purification of the Tyr701 *p*CMF Mutant STAT1. The human STAT1 gene in plasmid pXC101 was a gift from Xiaomin Chen at the University of Texas M.D. Anderson Cancer Center. The Tyr701 → TAG mutation was introduced by QuikChange mutagenesis (Stratagene) according to the manufacturer's protocol. The STAT1 fragment (132–712) with the Tyr701 → TAG mutation was inserted into the *Spe*I and *Bgl*II sites of plasmid pLEI (*35*) to afford plasmid pSTAT701TAG, which encodes the Tyr701 → TAG mutant of STAT1 under the control of a bacteriophage *T5* promoter and t_0 terminator, the *Mj*tRNA$^{Tyr}_{CUA}$ gene under the control of the *lpp* promoter and *rrnC* terminator, a chloramphenicol resistance marker, and a p15a origin. Plasmid pBK-*p*CMFRS#1 encodes the *p*CMF-specific *Mj*TyrRS mutant under the control of the constitutive *E. coli* GlnRS promoter and terminator. *E. coli* BL21 (DE3) competent cells were cotransformed with pSTAT701TAG and pBK-*p*CMFRS#1 and were grown in 500 mL of minimal media containing 1% glycerol and 0.3 mM leucine (GMML medium) with 50 μg mL^{-1} kanamycin, 34 μg mL^{-1} chloramphenicol, and 1.0 mM *p*CMF at 37 °C. When the OD_{600} reached 0.5, the culture was transferred to a 25 °C shaker and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM to induce protein expression. Cells were grown for an additional 16 h at 25 °C, pelleted by centrifugation (8 min; 4 °C, 8000*g*), and resuspended in 10 mL of ice cold extraction buffer [20 mM HEPES·HCl, pH 7.6, 0.1 M KCl, 10% glycerol, 1 mM EDTA, 10 mM $MnCl_2$, 20 mM DTT, and tablets of Complete (protease inhibitor cocktail tablets, Roche Applied Science, 1 tablet for 50 mL of solution)]. The cells were lysed by sonication and pelleted again (45 min; 4 °C, 27,500*g*). The supernatant was isolated and polyethylenimine was added (0.1% final; Sigma). The solution was gently mixed and then centrifuged for 15 min at 15,000*g*. The soluble STAT1 mutant in the supernatant was precipitated with saturated ammonium sulfate solution (Sigma) in two steps (0–35%, 35–55% saturation) as described for the purification of wild-type STAT1 (*34*). The 35–55% pellet was redissolved in 3 mL of buffer (20 mM Tris·HCl, pH 9.0, 20 mM NaCl, 0.5 mM EDTA, 2 mM DTT, Roche Complete protease inhibitor cocktail tablet) and dialyzed against the same buffer. The sample was then applied to an anion-exchange column (Mono Q HR 10/10 column, Amersham Biosciences) previously equilibrated with buffer A (20 mM Tris·HCl, 2 mM DTT, pH 9.0). The proteins were eluted with a linear gradient of 0–0.3 M NaCl. Peak fractions were analyzed by SDS-PAGE on an 8% PAGE gel. Fractions from a major peak that eluted at 0.18 M NaCl were pooled together and subjected to gel filtration (Superdex 200 HR 10/30 column, Amersham Biosciences). Proteins were eluted with 20 mM Tris·HCl, 100 mM NaCl, 2 mM DTT, pH 8.0. The final purified Tyr701 *p*CMF STAT1 (132–712) mutant was pooled and dialyzed against 10 mM Tris·HCl, 25 mM NaCl, 2 mM DTT, pH 8.0. The purified protein was concentrated to ~20 mg mL^{-1} using a Microcon YM-3 centrifugal filter device (3000 MWCO; Millipore), quick frozen on dry ice, and stored at −70 °C. The concentration of protein was measured by Bradford assay (BCA kit, Biorad).

^{32}P Labeling of M67 Duplex. Two complementary M67-site-containing oligonucleotides with the following sequences (M67 site underlined) were PAGE-purified: JX178, 5′-TCCACAGTTTCCCGTAAAT-GC-3′; JX179, 3′-AGGTGTCAAAGGGCATTTACG-5′. JX178 was dissolved at a concentration of 4 μM in annealing buffer (5 mM Tris·HCl, 50 mM KCl, 10 mM $MgCl_2$, pH 8.0), and 10 pmol of this oligonucleotide was ^{32}P-labeled with T4 PNK (50 pmol of [γ-^{32}P]ATP, 20 U of T4 PNK from New England Biolabs, Ipswich, MA). The T4 PNK was inactivated for 20 min at 65 °C. Labeled JX178 was mixed at 1:2 ratio with JX179 and annealed (94 °C for 1 min followed by cooling slowly to RT). Free [γ-^{32}P]ATP was removed by a Qiagen Nucleotide Removal kit. Labeled oligonucleotides were stored at 4 °C.

EMSA. Radiolabeled DNA (at a concentration of 0.3 nM) was mixed with the indicated amount of the Tyr701 *p*CMF mutant STAT1 (132–712) in a 12.5 μL reaction volume that contained 50 ng of dIdC and 2.5 μg of bovine serum albumin (Boehringer Mannheim) in DNA binding buffer (20 mM HEPES·HCl, 4% Ficoll, 40 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM DTT, pH 8.0) (*34*). The reaction mixture was incubated at RT for 30 min and then resolved on a 4–20% Novex TBE gel (Invitrogen), which had been pre-run for 2 h at ~20 V cm^{-1} at 4 °C. Electrophoresis was continued for 48 min at 4 °C. After samples were loaded, electrophoresis was performed for an additional 48 min at 4 °C. The gel was exposed to a Molecular Dynamics PhosphorImager phosphor screen and subsequently scanned on a Storm 860 instrument. Intensities of bands corresponding to free and protein-bound duplex DNA were determined by using the ImageQuant software. To calculate K_D, the amount of protein-bound oligonucleotide was expressed as a percentage of the total amount of oligonucleotide present in the reaction and plotted against the concentration of protein in the reaction. Thus, obtained data points were fitted with a 4-parameter logistic function, and K_{app} was then calculated as the protein concentration at which 50% of oligonucleotide was in the protein-bound state.

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Supporting Information Available: This material is free of charge *via* the Internet.

REFERENCES

1. Feng, G. S., Hui, C. C., and Pawson, T. (1993) SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases, *Science 259*, 1607–1611.
2. Tailor, P., Gilman, J., Williams, S., Couture, C., and Mustelin, T. (1997) Regulation of the low molecular weight phosphotyrosine phosphatase by phosphorylation at tyrosines 131 and 132, *J. Biol. Chem. 272*, 5371–5374.
3. Domchek, S. M., Auger, K. R., Chatterjee, S., Burke, T. R., Jr., and Shoelson, S. E. (1992) Inhibition of SH2 domain/phosphoprotein association by a nonhydrolyzable phosphonopeptide, *Biochemistry 31*, 9865–9870.
4. Zhang, Z., Shen, K., Lu, W., and Cole, P. A. (2003) The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored *via* expressed protein ligation, *J. Biol. Chem. 278*, 4668–4674.
5. Lu, W., Gong, D., Bar-Sagi, D., and Cole, P. A. (2001) Site-specific incorporation of a phosphotyrosine mimetic reveals a role for tyrosine phosphorylation of SHP-2 in cell signaling, *Mol. Cell 8*, 759–769.
6. Lu, W., Shen, K., and Cole, P. A. (2003) Chemical dissection of the effects of tyrosine phosphorylation of SHP-2, *Biochemistry 42*, 5461–5468.
7. Dawson, P. E., and Kent, S. B. (2000) Synthesis of native proteins by chemical ligation, *Annu. Rev. Biochem. 69*, 923–960.
8. Evans, T. C., Jr., and Xu, M. Q. (1999) Intein-mediated protein ligation: harnessing nature's escape artists, *Biopolymers 51*, 333–342.
9. Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001) Expanding the genetic code of *Escherichia coli*, *Science 292*, 498–500.
10. Chin, J. W., Cropp, T. A., Anderson, J. C., Mukherji, M., Zhang, Z., and Schultz, P. G. (2003) An expanded eukaryotic genetic code, *Science 301*, 964–967.

11. Zhang, Z., Alfonta, L., Tian, F., Bursulaya, B., Uryu, S., King, D. S., and Schultz, P. G. (2004) Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells, *Proc. Natl. Acad. Sci. U.S.A. 101*, 8882–8887.
12. Wang, L., Xie, J., and Schultz, P. G. (2006) Expanding the genetic code, *Annu. Rev. Biophys. Biomol. Struct. 35*, 225–249.
13. Xie, J., and Schultz, P. G. (2006) A chemical toolkit for proteins—an expanded genetic code, *Nat. Rev. Mol. Cell Biol. 7*, 775–782.
14. Burke, T. R., Jr., Yao, Z. J., Liu, D. G., Voigt, J., and Gao, Y. (2001) Phosphoryltyrosyl mimetics in the design of peptide-based signal transduction inhibitors, *Biopolymers 60*, 32–44.
15. Gilmer, T., Rodriguez, M., Jordan, S., Crosby, R., Alligood, K., Green, M., Kimery, M., Wagner, C., Kinder, D., and Charifson, P. (1994) Peptide inhibitors of src SH3-SH2-phosphoprotein interactions, *J. Biol. Chem. 269*, 31711–31719.
16. Burke, T. R., Jr., Luo, J., Yao, Z. J., Gao, Y., Zhao, H., Milne, G. W., Guo, R., Voigt, J. H., King, C. R., and Yang, D. (1999) Monocarboxylic-based phosphotyrosyl mimetics in the design of GRB2 SH2 domain inhibitors, *Bioorg. Med. Chem. Lett. 9*, 347–352.
17. Tong, L., Warren, T. C., Lukas, S., Schembri-King, J., Betageri, R., Proudfoot, J. R., and Jakes, S. (1998) Carboxymethyl-phenylalanine as a replacement for phosphotyrosine in SH2 domain binding, *J. Biol. Chem. 273*, 20238–20242.
18. Waksman, G., Kominos, D., Robertson, S. C., Pant, N., Baltimore, D., Birge, R. B., Cowburn, D., Hanafusa, H., Mayer, B. J., Overduin, M., Resh, M. D., Rios, C. B., Silverman, L., and Kuriyan, J. (1992) Crystal structure of the phosphotyrosine recognition domain SH2 of v-src complexed with tyrosine-phosphorylated peptides, *Nature 358*, 646–653.
19. Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D., and Kuriyan, J. (1993) Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms, *Cell 72*, 779–790.
20. Hao, M., Lowy, A. M., Kapoor, M., Deffie, A., Liu, G., and Lozano, G. (1996) Mutation of phosphoserine 389 affects p53 function *in vivo*, *J. Biol. Chem. 271*, 29380–29385.
21. Mehl, R. A., Anderson, J. C., Santoro, S. W., Wang, L., Martin, A. B., King, D. S., Horn, D. M., and Schultz, P. G. (2003) Generation of a bacterium with a 21 amino acid genetic code, *J. Am. Chem. Soc. 125*, 935–939.
22. Xie, J., and Schultz, P. G. (2005) An expanding genetic code, *Methods 36*, 227–238.
23. Turner, J. M., Graziano, J., Spraggon, G., and Schultz, P. G. (2005) Structural characterization of a *p*-acetylphenylalanyl aminoacyl-tRNA synthetase, *J. Am. Chem. Soc. 127*, 14976–14977.
24. Yamane, T., Miller, D. L., and Hopfield, J. J. (1981) Discrimination between D- and L-tyrosyl transfer ribonucleic acids in peptide chain elongation, *Biochemistry 20*, 7059–7064.
25. Heckler, T. G., Roesser, J. R., Xu, C., Chang, P. I., and Hecht, S. M. (1988) Ribosomal binding and dipeptide formation by misacylated tRNA(Phe),S, *Biochemistry 27*, 7254–7262.
26. Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., and Schultz, P. G. (1989) A general method for site-specific incorporation of unnatural amino acids into proteins, *Science 244*, 182–188.
27. Bain, J. D., Diala, E. S., Glabe, C. G., Wacker, D. A., Lyttle, M. H., Dix, T. A., and Chamberlin, A. R. (1991) Site-specific incorporation of nonnatural residues during *in vitro* protein biosynthesis with semi-synthetic aminoacyl-tRNAs, *Biochemistry 30*, 5411–5421.
28. Dedkova, L. M., Fahmi, N. E., Golovine, S. Y., and Hecht, S. M. (2003) Enhanced D-amino acid incorporation into protein by modified ribosomes, *J. Am. Chem. Soc. 125*, 6616–6617.
29. Dedkova, L. M., Fahmi, N. E., Golovine, S. Y., and Hecht, S. M. (2006) Construction of modified ribosomes for incorporation of D-amino acids into proteins, *Biochemistry 45*, 15541–15551.
30. Kobayashi, T., Nureki, O., Ishitani, R., Yaremchuk, A., Tukalo, M., Cusack, S., Sakamoto, K., and Yokoyama, S. (2003) Structural basis for orthogonal tRNA specificities of tyrosyl-tRNA synthetases for genetic code expansion, *Nat. Struct. Biol. 10*, 425–432.
31. Pogulis, R. J., Vallejo, A. N., and Pease, L. R. (1996) *In vitro* recombination and mutagenesis by overlap extension PCR, *Methods Mol. Biol. 57*, 167–176.
32. Darnell, J. E., Jr. (1997) STATs and gene regulation, *Science 277*, 1630–1635.
33. Chen, X., Vinkemeier, U., Zhao, Y., Jeruzalmi, D., Darnell, J. E., Jr., and Kuriyan, J. (1998) Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA, *Cell 93*, 827–839.
34. Vinkemeier, U., Cohen, S. L., Moarefi, I., Chait, B. T., Kuriyan, J., and Darnell, J. E., Jr. (1996) DNA binding of *in vitro* activated Stat1 alpha, Stat1 beta and truncated Stat1: interaction between NH2-terminal domains stabilizes binding of two dimers to tandem DNA sites, *EMBO J. 15*, 5616–5626.
35. Wang, L., Zhang, Z., Brock, A., and Schultz, P. G. (2003) Addition of the keto functional group to the genetic code of *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 100*, 56–61.