Incorporation of the Unnatural Amino Acid *p*-benzoyl-*L*-phenylalanine (Bpa) into a G Protein-coupled Receptor in its Native Context

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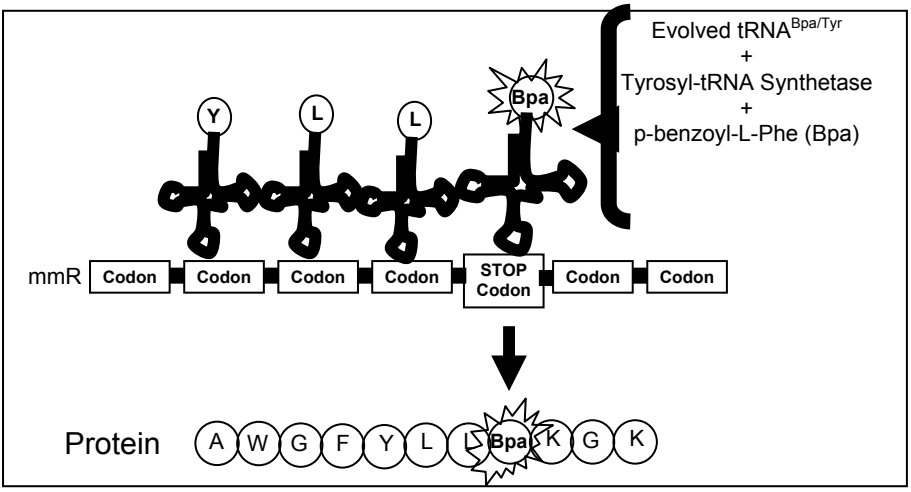
Introduction

Ligand binding initiates a change in the conformation of G protein-coupled receptors (GPCRs) resulting in activation of the G protein-mediated signal transduction cascade [1]. We are studying a novel approach to elucidate the dynamics of GPCR structure by the co-translational introduction of unnatural amino acids (UAAs) into the receptor.

UAAs can be synthesized to contain a variety of chemical moieties for use as photoaffinity labels, fluorescent labels, or spectroscopic probes. Orthogonal tRNA/aminoacyl-tRNA synthetase pairs evolved and expressed in the target cell have been used to incorporate UAAs into heterologously expressed protein in living cells [2]. The mutated tRNA, charged with its UAA, recognizes the amber TAG stop codon and incorporates the non-natural amino acid into the nascent polypeptide chain (See Figure below). UAAR has been widely used in the heterologous *Xenopus* oocyte expression system to insert UAAs into a variety of receptors and channel proteins. Thus far the genetic incorporation of UAAs into an integral membrane protein in its native eukaryotic host cell has not been accomplished.

Here we report the site-specific incorporation of *p*-benzoyl-*L*-phenylalanine (Bpa) into Ste2p, the prototypical yeast GPCR, using an orthologous tRNA/aminoacyl tRNA synthetase pair (See Figure Below).



S. Del Valle et al. (eds.), *Peptides for Youth: The Proceedings of the 20th American Peptide Symposium*, 333
DOI: 10.1007/978-0-387-73657-0_149,

The TAG stop codon was engineered into specific sites within the *STE2* coding region. The cells accumulated Bpa from the growth medium either as the free amino acid analog or as a methionyl-Bpa peptide. Upon translation, Bpa was incorporated into the nascent Ste2p and ultimately expressed at the cell surface.

Results and Discussion

Eight different TAG stops were inserted into the *STE2* coding sequence by site-directed mutagenesis to create Ste2p-F55^{TAG}, Ste2p-S107^{TAG}, etc. (Table 1). To assay the sensitivity of the mutant receptors to α-factor, cells were grown with and without Bpa, and the amount of pheromone necessary to generate a 20 mm diameter halo was determined (Table 1).

Table 1. Pheromone required (μg) for 20 mm diameter halo for TAG mutants.

	WT	F55	S107	G115	V127	G188	Y193	F204	Y266
- Bpa	0.18	0.58	0.74	0.35	0.70	-	-	0.96	-
+ Bpa*	0.28	-	8	1.54	2.3	5.9	0.32	-	-

** Bpa concentration was 2 mm. Absence of halo is indicated by a dash (-)*

Upon binding pheromone to native Ste2p a signal transduction cascade is activated and growth arrest of the cell occurs, resulting in a clear zone or "halo"around a disk impregnated with α-factor. In the absence of Bpa, the tRNA should not be charged, resulting in a truncated Ste2p which will not respond to pheromone. In the presence of Bpa, the TAG codon would be recognized by the charged tRNA, and full-length protein should be synthesized.

The G188^{TAG} and Y193^{TAG} receptors did not respond to pheromone in the absence of Bpa, but growth arrest did occur in the presence of Bpa, although the G188 receptor did not respond as well as the Y193 receptor. (5.9 μg vs. 0.32 μg, respectively). This indicates that Bpa incorporation resulted in functional proteins Ste2p-G188Bpa and Ste2p-Y193Bpa For the S107^{TAG}, G115^{TAG} and V127^{TAG} receptors halos formed in the absence of Bpa (0.74, 0.35, and 0.70 μg per disk respectively), indicating that there was some "read-through" of the amber stop codon in the absence of Bpa. Read-through most often occurs when the context of the nucleotides flanking the stop codon is not optimal for termination [3]. Halos formed in the presence of Bpa, but the receptors were less sensitive and required more pheromone compared to the wild-type receptor (S107 ^{TAG} - 8 μg, G115^{TAG} - 1.5 μg and V127^{TAG} - 2.3 μg per disk) to generate a 20 mm halo. For the F55^{TAG} and F204^{TAG} mutants, the receptors were still active in the absence of Bpa addition, however in the presence of Bpa, no halos were observed, suggesting that under these conditions Bpa was incorporated into Ste2p, but the amino acid analog was not tolerated at those positions. The Y266^{TAG} receptor was not functional in either the presence or absence of Bpa, further supporting the essential role of the Y266 residue in signal transduction, as has been noted previously [4].

As determined by immunblot analysis wild-type Ste2p was synthesized at full length in both the presence and absence of Bpa and could be detected by antibodies directed against either the N-terminus or C-terminus of the protein. In contrast, the receptors encoded by F55^{TAG} and G188^{TAG} were detected only in the presence of Bpa. For the Y193^{TAG} mutant, a low level of expression was detected in the absence of Bpa, and was enhanced in the presence of Bpa. C-terminally truncated forms of

the receptor were observed for the $G188^{TAG}$ and $Y193^{TAG}$ encoded receptors using antibody to the N-terminus. Truncated forms of the $F55^{TAG}$ receptor were not detected with the N-terminal antibody. Expression of the full length $F55^{TAG}$ Bpa-containing receptor was enhanced by increasing the Bpa concentration in the growth medium. This suggested that entry of Bpa into the cell is important in determining the efficiency of Bpa insertion into the protein. Cell surface expression of the receptors encoded by $F55^{TAG}$, $G188^{TAG}$, and $Y193^{TAG}$ grown in the presence of Bpa was also confirmed by whole-cell saturation binding analysis using radiolabeled α-factor.

To improve delivery of Bpa into the cell we synthesized the dipeptide Met-Bpa and used it as a source of Bpa during cell growth. Small peptides enter yeast cells across the di-/tripeptide transporter Ptr2p [5, 6] and are hydrolyzed to free amino acids by intracellular peptidases. In the absence of Bpa, $Y193^{TAG}$ mutant was minimally expressed as detemined by immunotblot, while in the presence of Met-Bpa (0.1 mM) full length protein was detected at a level exceeding the expression observed when grown on free Bpa (0.1 mM).

MALDI-TOF mass spectrum analysis of purified wild-type and receptor encoded by $G188^{TAG}$ was performed to determine whether incorporation of Bpa was achieved in the mutant receptor. The $G188^{TAG}$ receptor was chosen based on the CNBr cleavage profile for Ste2p which facilitated mass spectroscopy analysis. The substitution of Bpa (269.30 Da) for glycine (75.07 Da) at position 188 resulted in a mass shift of 194 Da in the 188Bpa-containing peptide. This shift confirmed incorporation of Bpa into Ste2p at position 188.

The experiments reported are the first to our knowledge to incorporate an unnatural amino acid into a GPCR in its natural environment. We believe such methodology will provide a very rich source of experimental methodologies for studying the structure and function of these important membrane proteins.

Acknowledgments

This work was supported by grants NIH GM-22086(FN) and NIH GM-22087(JMB).

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