Unnatural Amino Acid Replacement in a Yeast G Protein-Coupled Receptor in Its Native Environment†

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ABSTRACT: Ste2p is the G protein-coupled receptor (GPCR) for the tridecapeptide pheromone α factor of *Saccharomyces cerevisiae*. This receptor−pheromone pair has been used extensively as a paradigm for investigating GPCR structure and function. Expression in yeast harboring a cognate tRNA/aminoacyl-tRNA synthetase pair specifically evolved to incorporate *p*-benzoyl-L-phenylalanine (Bpa) in response to the amber codon allowed the biosynthesis of Bpa-substituted Ste2p in its native cell. We replaced natural amino acid residues in Ste2p with Bpa by engineering amber TAG stop codons into *STE2* encoded on a plasmid. Several of the expressed Bpa-substituted Ste2p receptors exhibited high-affinity ligand binding, and incorporation of Bpa into Ste2p influenced biological activity as measured by growth arrest of whole cells in response to α factor. We found that, at concentrations of 0.1−0.5 mM, a dipeptide containing Bpa could be used to enhance delivery of Bpa into the cell, while at 2 mM, both dipeptide and Bpa were equally effective. The application of a peptide delivery system for unnatural amino acids will extend the use of the unnatural amino acid replacement methodology to amino acids that are impermeable to yeast. Incorporation of Bpa into Ste2p was verified by mass spectrometric analysis, and two Bpa-Ste2p mutants were able to selectively capture α factor into the ligand-binding site after photoactivation. To our knowledge, this is the first experimental evidence documenting an unnatural amino acid replacement in a GPCR expressed in its native environment and the use of a mutated receptor to photocapture a peptide ligand.

G protein-coupled receptors (GPCRs)[1] are activated upon binding their cognate ligands. Ligand binding initiates a change in the conformation of these integral membrane proteins. This promotes signal transduction across the membrane and the activation of the G protein-mediated signal transduction cascade (*1, 2*). The interactions between ligands and their receptors are defined for a number of GPCRs (*3*). However, the ligand-induced changes in protein structure, which initiate signal transduction, are difficult to study. Although site-directed mutagenesis has been used to address the issue of GPCR activation, more methods for studying ligand-dependent conformational changes upon receptor activation are needed. One promising approach, which may be adapted to the study of the dynamics of the GPCR structure is the use of orthogonal pairs of tRNA/aminoacyl-tRNA synthetases evolved and expressed in the target cell to incorporate unnatural amino acids (*4–7*).

Non-naturally occurring amino acids can be synthesized to contain a variety of chemical moieties for use as photoaffinity labels or fluorescent and/or spectroscopic probes. To autonomously incorporate unnatural amino acids into proteins in living cells, the mutated tRNA is designed to recognize a specific codon, usually a nonsense codon, such as the amber TAG stop. Provided that there is a sufficient quantity of the unnatural amino acid in the cytoplasm, the evolved cognate aminoacyl-tRNA synthetase specifically charges its orthogonal tRNA with this novel amino acid for delivery into the protein.

The genetic codes of *Escherichia coli*, yeast, and mammalian cells have been altered to allow for the translational insertion of more than 30 unnatural amino acids with a variety of novel properties useful in the study of protein structure and function (*5, 8*). Thus far, these applications have focused primarily on soluble proteins. With respect to eukaryotic membrane proteins expressed in mammalian cells, unnatural amino acids have been introduced into the nicotinic

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[1] Abbreviations: GPCR, G protein-coupled receptor; G protein, heterotrimeric GTP-binding protein; Ste2p, α-factor receptor encoded by the *STE2* gene; Bpa, *p*-benzoyl-L-phenylalanine; MLWU, medium lacking tryptophan and uracil; SDS, sodium dodecyl sulfate; Nle, norleucine; standard one-letter abbreviations for amino acids are used.

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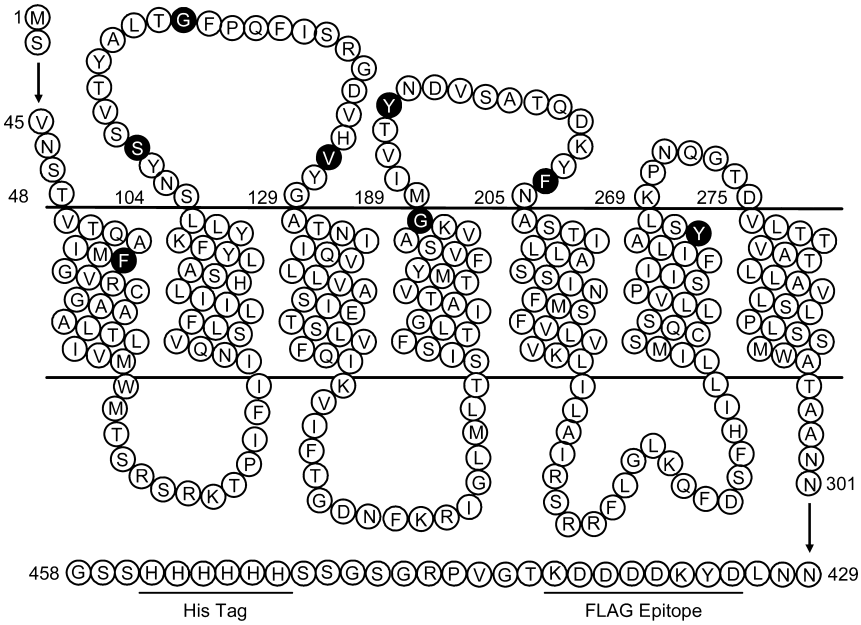

FIGURE 1: Sites targeted for Bpa insertion into Ste2p. The coding region of the *STE2* gene was modified to introduce the amber TAG codon, resulting in the insertion of Bpa into Ste2p at the sites indicated in black (F55, S107, G115, V127, G188, Y193, F204, and Y266). The FLAG and His tags are underlined.

acetycholine receptor in both CHO and cultured neuronal cells (*9*) and in the epidermal growth factor receptor in HEK293 cells (*10*). A heterologous *Xenopus* oocyte expression system has also been used extensively to insert non-natural amino acids into the nicotinic acetylcholine receptor (*11*) the neurokinin-2 receptor (*12*), the $GABA_A$ receptor (*13*), the NMDA receptor (*14*), and numerous channel proteins, including the serotonin-gated ion channel (*15*), the inwardly rectifying potassium channel (*16*), and the voltage-sensitive sodium channel (*17*). In both the mammalian and *Xenopus* expression systems, a tRNA chemically charged with the non-natural amino acid is introduced into the cell along with the target mRNA. While these systems are useful for the short-term studies of protein properties, they are not amenable to large-scale production of proteins into which unnatural amino acids have been incorporated. Recently, it was demonstrated in CHO cells that unnatural amino acids could be introduced into green fluorescent protein (GFP) using specific orthogonal tRNA/aminoacyl-tRNA synthetase pairs (*6*). Because the yeast *Saccharomyces cerevisiae* provides a tractable system in which to develop this methodology, we report here the genetic incorporation of an unnatural amino acid into a polytopic membrane protein in its native eukaryotic host cell.

To date in *S. cerevisiae*, orthologous tRNA/aminoacyl-tRNA synthetase pairs have been used to incorporate a variety of unnatural amino acids, including the photoactivatable amino acid analogue *p*-benzoyl-L-phenylalanine (Bpa) (*4*), the fluorescent amino acid dansyl alanine (*18*), and acetylene- and azide-containing amino acids (*7, 19*), into the soluble protein human superoxide dismutase. Here, we report the site-specific incorporation of Bpa into Ste2p, a yeast GPCR, using an orthologous tRNA/aminoacyl-tRNA synthetase pair. In this system, the amber TAG stop codon was engineered into specific sites within the *STE2* coding region. Bpa was supplied to the cells either as the free amino acid analogue or as the dipeptide Met−Bpa. Upon translation of the message, Bpa was incorporated into the nascent Ste2p and ultimately expressed at the cell surface. Two of our Bpa−Ste2p receptors were used to selectively photocapture biotinylated α factor. To our knowledge, this is the first report of the expression in the native host cell of a GPCR containing a photoactivatable amino acid. The successful photocapture of α factor by novel Ste2p mutants shows that GPCRs containing unnatural amino acids can be used to capture ligand and study changes in domain−domain interactions during GPCR activation.

EXPERIMENTAL PROCEDURES

Media, Reagents, Strains, and Plasmids. S. cerevisiae strain DK102 (*MAT***a**, *ura3−52 lys2−801^{am} ade201^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1 ste2*::*HIS3 sst1-Δ5*) was used for growth arrest and binding assays, and the protease-deficient strain BJS21 (*MAT***a**, *prc1−407 prb1−1122 pep4−3 leu2 trp1 ura3−52 ste2*::*Kan^R*) was used for protein isolation and immunoblot analysis. C-Terminal FLAG and His-tagged STE2 was PCR-amplified from plasmid pNED1 (*20*) and cloned into the plasmid p426-GPD (*21*) to yield plasmid pCL01. The plasmid pCL01 was engineered by single-stranded mutagenesis to incorporate TAG stop codons at eight specific positions within the *STE2* coding region (Figure 1). The sequence of all TAG mutants was verified by DNA sequence analysis completed by the Molecular Biology Resource Facility located on the campus of the University of Tennessee. Mutagenic and sequencing primers were purchased from Sigma/Genosys (The Woodlands, TX) or IDT (Coralville, IA). The pCL01 TAG mutant plasmids were cotransformed by the method of Geitz (*22*) into DK102 and BJS21 cells along with plasmid pECTyrRS/tRNACUA, encoding the orthogonal amber suppressor tRNA synthetase−tRNA pair genetically modified to allow for incorporation of *p*-benzoyl-L-phenylalanine (Bpa) (*4*). Transformants were selected by growth on minimal medium (*23*) lacking tryptophan and uracil (designated as MLWU) to maintain selection for the plasmids. Cells used in the assays described

below were cultured in MLWU and grown to mid-log phase at room temperature with shaking (200 rpm) in the presence or absence of 2 mM Bpa, unless otherwise specified. Methionyl-Bpa was synthesized by standard solution phase techniques (*24*) and purified by reverse-phase high-performance liquid chromatography (HPLC) to greater than 99% homogeneity. All media components were obtained from BD (Franklin Lakes, NJ) and were of the highest quality available. Bpa was purchased from Bachem (Torrance, CA) and was dissolved in NaOH (1 N) at a final concentration of 100 mM immediately before use.

Growth Arrest Assays. DK102 cells expressing the wild-type (WT) or TAG constructs were grown at 30 °C in MLWU, harvested, washed 3 times with water, and resuspended at a final concentration of 5×10^6 cells/mL. Cells (1 mL) were combined with 3.5 mL agar noble (1.1%) with or without the addition of Bpa (2 mM final concentration) and poured as a top agar lawn onto MLWU medium. Filter disks (BD, Franklin Lakes, NJ) impregnated with the tridecapeptide pheromone α factor (WHWLQLKPGQPNle[12]Y) synthesized and characterized as previously described (*25*) were placed on the top agar, and the plates were incubated at room temperature (23 °C) for 48−72 h. The growth arrest assays were repeated a minimum of 3 times, and similar results were observed for each replicate.

Immunoblots. BJS21 cells expressing WT or TAG-*STE2* constructs grown in the presence or absence of Bpa were used to prepare total cell membranes isolated as previously described (*20*). The protein concentration was determined (BioRad, Hercules, CA), and membranes were solubilized in sodium dodecyl sulfate (SDS) sample buffer. Proteins (2 μg/lane for WT and 30 μg/lane for mutants) were fractioned by SDS−polyacrylamide gel electrophoresis (PAGE) and immunoblotted. Blots were probed with FLAG antibody (Sigma/Aldrich Chemical, St. Louis, MO) or an antibody directed against the N-terminal 100 amino acids of Ste2p generously provided by J. Konopka (*26*). The immunoblots were imaged, and band density was quantitated using Quantity One software (version 4.5.1) on a Chemi-Doc XRS photodocumentation system (BioRad, Hercules, CA). Multiple repeats of immunoblot experiments yielded the same results.

Binding Assays. Tritiated α factor (10.2 Ci/mmol, 12 μM) prepared as previously described (*25*) was used in saturation binding assays on whole cells. DK102 cells expressing WT or TAG constructs were harvested, washed 3 times with YM1 (*20*), and adjusted to a final concentration of 2×10^7 cells/mL. Cells (600 μL) were combined with 150 μL of ice-cold 5× binding medium (YM1 plus protease inhibitors [YM1i (*20*)] supplemented with [^{3}H]α factor) and incubated at room temperature for 30 min. The final concentration of [^{3}H]α factor ranged from 0.5×10^{-10} to 1×10^{-6} M. Upon completion of the incubation interval, 200 μL aliquots of the cell−pheromone mixture were collected in triplicate and washed over glass fiber filter mats using the Standard Cell Harvester (Skatron Instruments, Sterling, VA). Retained radioactivity on the filter was counted by liquid scintillation spectroscopy. DK102 cells lacking Ste2p were used as a nonspecific binding control for the assays. Binding assays were repeated a minimum of 3 times, and similar results were observed for each replicate. Specific binding for each mutant receptor was calculated by subtracting the nonspecific values from those obtained for total binding. Specific binding data were analyzed by nonlinear regression analysis for single-site binding using Prism software (GraphPad Software, San Diego, CA) to determine the K_m (in nanomolars) and B_{max} values (receptors/cell) for each mutant receptor.

Matrix-Assisted Laser Desorption Ionization−Time of Flight (MALDI−TOF). BJS21 cells expressing either the WT or G188^{TAG} Ste2p were grown in the presence or absence of Bpa and used to prepare total cell membranes as previously described (*20*). Approximately 3 mg of cell membranes was resuspended in ice-cold solubilization buffer [50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100] with protease inhibitors (PMSF, pepstatin A, and leupeptin), incubated overnight at 4 °C with end-over-end mixing, and then centrifuged at 15000*g* for 30 min to remove nonsoluble material. The solubilized proteins were then mixed with FLAG resin (Sigma/Aldrich Chemical Co., St. Louis, MO) and incubated at 4 °C with end-over-end mixing for 6 h. The resin was collected by centrifugation at low speed (800*g* for 1 min) and resuspended and collected 4 times in TBS buffer (25 mM Tris-HCl, 140 mM NaCl, and 3 mM KCl at pH 7.4). Ste2p was eluted by resuspending the resin in 1 mL of ice-cold elution buffer (0.1 M glycine HCl at pH 3.5) and incubated at 4 °C with end-over-end mixing for 5 min. The resin was pelleted by centrifugation (2000*g* for 1 min), and the supernatant, containing the eluted Ste2p, was transferred to a fresh tube containing 20 μL of 0.5 M Tris-HCl at pH 7.4 and 1.5 M NaCl. Purity and concentration of samples was estimated by Coomassie Blue and silver staining of SDS−PAGE gels. The samples were also analyzed by immunoblotting using an antibody to the FLAG epitope on the C terminus of Ste2p.

Samples containing eluted Ste2p (∼50 μg) were dried by vacuum centrifugation (Thermo Scientific, Waltham, MA) and then dissolved in 100% trifluoroacetic acid (TFA) containing 10 mg/mL cyanogen bromide (CNBr). Deionized−distilled water (ddH_2O) was then added to adjust the final TFA concentration to 70%, and the sample was incubated at 37 °C in the dark for 18 h. The samples were dried by vacuum centrifugation and washed 3 times with ddH_2O and resuspended in 0.1% TFA. The resulting CNBr peptide fragments were further washed and concentrated using ZipTip pipet tips (Millipore Corporation, Billerica, MA) following the directions of the manufacturer and resuspended in 70% acetonitrile/30% water (0.1% TFA). For MALDI−TOF analysis, α-cyano-4-hydroxy-*trans*-cinnamic acid (ACHA, Sigma/Aldrich Chemical Co., St. Louis, MO) at a concentration of 20 mg/mL in 50% acetonitrile/50% water (0.1% TFA) was used as the matrix. The digested samples (0.5 μL eluate from ZipTip) were either mixed with 0.5 μL of matrix before spotting on the target or 1.0 μL of matrix was spotted and allowed to dry before applying 1.0 μL of samples. MADLI−TOF spectra were acquired on a Bruker Daltonics (Boston, MA) Microflex using both reflector and linear methods.

Cross-linking. A biotinylated form of α factor, [K[7] (biotinylamidocaproate), Nle[12]]α factor, was prepared as previously described (*27*). Membranes prepared from BJS21 cells expressing the F55^{TAG}, G188^{TAG}, and Y193^{TAG} receptors, grown at room temperature in the presence or absence of Bpa (1 mM), were suspended in PPBi buffer [0.5 M potassium phosphate at pH 6.2, 10 mM tert-amyl methyl

ether (TAME), 10 mM sodium azide, 10 mM potassium fluoride, and 0.1% bovine serum albumin (BSA)], incubated with biotinylated pheromone (1 μM) in the presence or absence of nonbiotinylated pheromone (100 μM) for 30 min at room temperature, and then chilled to 4 °C for the remainder of the procedure. Cross-linking was performed as previously described (*27*). Briefly, the membranes were exposed to UV light at 365 nm using a Stratalinker (Stratagene, La Jolla, CA) for three 15 min intervals. The cross-linked membranes were washed 3 times with CAPS buffer [10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (Sigma, St. Louis, MO) at pH 10] by centrifugation to remove noncross-linked biotinylated α factor, fractionated by SDS−PAGE and immunoblotted with antibody to Ste2p and with Neutravidin−horseradish peroxidase (HRP) conjugate (Pierce, Rockford, IL) to detect the biotinylated pheromone covalently linked to the receptor. The signal generated by the Neutravidin−HRP conjugate associated with the biotinylated ligand−receptor complex was quantitated by measurement of the band density using Quantity One software (version 4.5.1) on a Chemi-Doc XRS photodocumentation system (BioRad, Hercules, CA). Before fractionation by SDS−PAGE, the amount of protein in each sample was determined, and amounts specified in the text for each experiment were loaded into each lane.

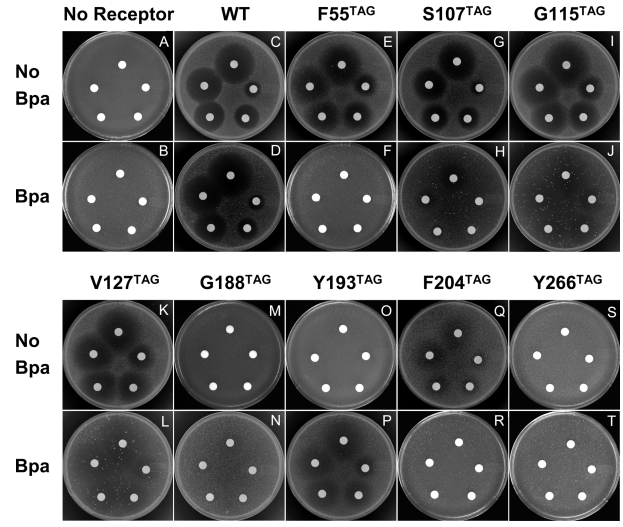

FIGURE 2: Halo assays of WT and Bpa mutant receptors. Cells lacking receptor (A and B), cells containing WT Ste2p (C and D), or cells with mutated Ste2p (E−T) were plated onto medium in the presence and absence of Bpa. Disks containing α factor (2, 1, 0.5, 0.2, and 0.1 μg/disk, starting at the 12 O'clock position on each plate and moving counter-clockwise) were placed onto the lawn of cells. The plates were incubated for 48−72 h at room temperature.

RESULTS

Effect of Bpa Incorporation on Ste2p Expression and Signal Transduction. The amber stop codon TAG was inserted into the *STE2* coding sequence by site-directed mutagenesis at the eight sites indicated in Figure 1. On the basis of the latest information concerning Ste2p topology (*28–30*), the sites were located in the first transmembrane domain ($F55^{TAG}$), in the first ($S107^{TAG}$, $G115^{TAG}$, and $V127^{TAG}$) and second ($Y193^{TAG}$ and $F204^{TAG}$) extracellular loops, at the border between the fourth transmembrane domain and the second loop ($G188^{TAG}$) and in the sixth transmembrane domain ($Y266^{TAG}$). These residues were selected on the basis of several criteria: (i) location, either in an extracellular loop or near the interface between a TM and an extracellullar surface, (ii) prior site-directed mutagenesis studies indicating that the residues (S107, G115, V127, and G188) were tolerant to amino acid substitution (*31*), and (iii) conservation of the aromatic functional group (F55, F204, and Y266) (*32, 33*). The plasmids bearing these mutations and the plasmid pECTyrRS/tRNACUA, which encoded the tRNA/tRNA synthetase pair, were transformed into DK102 and BJS21 cells. Cell membranes isolated from strain BJS21 expressing the TAG mutant constructs and the tRNA/tRNA synthetase pair were immunoblotted and probed with antibodies directed against the C-terminal FLAG epitope tag and the N-terminal 100 amino acids of Ste2p (*26*) to determine the effect of the amber stop codon on protein synthesis in the presence and absence of Bpa.

To determine if the Ste2p−Bpa mutant proteins were able to bind ligand and ultimately transduce the signal across the cell membrane, growth arrest or "halo" assays were conducted on these transformed cells. Disks impregnated with α factor were placed onto a top agar lawn of DK102 cells putatively expressing Ste2p−Bpa receptors, and the plates were incubated at room temperature for 48−72 h. The rationale to conduct these studies was 2-fold. In the absence of Bpa, the tRNA should not be charged, resulting in the production of truncated Ste2p. In this case, the cells should not respond to pheromone and growth arrest should not occur. In the presence of Bpa, the TAG codon should be suppressed by the charged tRNA and full-length protein should be synthesized. If the incorporation of Bpa results in expression, the mutant Ste2p proteins might bind pheromone, resulting in the initiation of signal transduction ultimately resulting in growth arrest, indicated by the formation of a clear halo around the disk.

For cells expressing WT Ste2p, dose-dependent halos with well-defined borders and clear zones of inhibition were formed in both the presence and absence of Bpa, indicating that Bpa addition to growth medium did not affect the pheromone response (parts C and D of Figure 2). Full-length WT Ste2p was synthesized in both the presence and absence of Bpa and could be detected by both N-terminal (Figure 3A) and C-terminal (Figure 3B) antibodies. Cells that were deleted for Ste2p did not form halos in either the presence or absence of Bpa (parts A and B of Figure 2), and Ste2p was not detected on immunoblots (see Figure S1 in the Supporting Information).

The mutants $F55^{TAG}$ (Figure 2E) and $F204^{TAG}$ (Figure 2Q) formed dose-dependent halos in the absence of Bpa, although these halos were "fuzzy", referring to the poor definition of the borders and reduced clarity of the zone of inhibition compared to the WT control. The fuzzy nature of these halos is most likely due to a decreased receptor number or to more rapid desensitization of the Bpa mutant receptor, resulting in a recovery from the growth arrest response (*34, 35*). Immunoblot analysis indicated that, in the absence of Bpa, the protein expression level of the $F55^{TAG}$ mutant was below the limit of detection (Figure 3A) and the expression of the $F204^{TAG}$ mutant was very low (see Figure S1 in the Supporting Information). Despite the inability to visualize the $F55^{TAG}$ protein on the immunoblot, sufficient protein was made by read-through expression to produce the biological

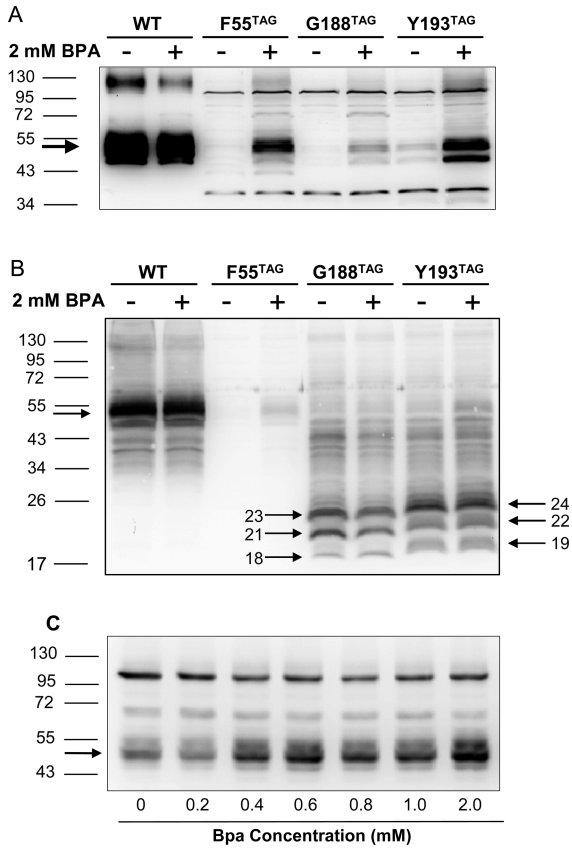

FIGURE 3: Immunoblots of membranes isolated from cells expressing Bpa mutant Ste2p. In each panel, the arrow at the left indicates the band corresponding to full-length Ste2p. (A) C-Terminal FLAG and (B) N-terminal anti-Ste2p immunoblots prepared from cells expressing the F55, G188, and Y193 receptors grown in the presence and absence of 2 mM Bpa. In B, the arrows in the lower portion of the figure indicate truncated forms of Ste2p. (C) FLAG immunoblot of membranes prepared from cells expressing the $F55^{TAG}$ receptor in the presence of increasing concentrations of Bpa.

response and to be detected by the ligand binding assay (see below). The observation that low levels of Ste2p are sufficient to result in signal transduction was made previously (*36*). These investigators reported that only a fraction of the receptors in a normal cell are necessary for signaling and that reduction of the receptor level to below 5% of the normal level led to smaller, turbid, fuzzy-edged growth arrest zones in response to α factor. The lack of receptor expression was required to completely block α-factor-induced growth arrest.

The presence of halos for the $F55^{TAG}$ and $F204^{TAG}$ receptors in the absence of Bpa indicates that there was "read-through" of the amber stop codon, resulting in the production of a functional receptor. In contrast, in the presence of Bpa, these same mutants did not form halos (parts F and R of Figure 2). Subsequent immunoblot analysis indicated that the expression of the $F55^{TAG}$ (Figure 3A) and $F204^{TAG}$ (see Figure S1 in the Supporting Information) receptors was enhanced in cells grown in the presence of Bpa, suggesting that Bpa was incorporated into Ste2p but was not tolerated at those positions, resulting in nonsignaling receptors. The expression was at relatively low levels; 15 times more protein was loaded for the mutants to allow for detection by the immunoblot compared to that of the WT (30 versus 2 μg).

Receptors with mutations at positions S107 (Figure 2G), G115 (Figure 2I), and V127 (Figure 2K) also exhibited read-through expression. Halos formed in the absence of Bpa, although they were fuzzy for the $G115^{TAG}$ and $V127^{TAG}$ receptors. Little or no protein was detected by immunoblot analysis for these three receptors when cells were grown in the absence of Bpa (see Figure S1 in the Supporting Information) indicating again, as in the case of $F55^{TAG}$, expression was below the limit of detection for the immunoblot even though sufficient protein was made by read-through expression to produce the biological response. In the presence of Bpa, expression of these three mutants resulted in halo formation, although the halos were not well-delineated (parts H, J, and L of Figure 2, respectively). Immunoblot analysis indicated that expression of these three proteins was enhanced in cells grown in the presence of Bpa (see Figure S1 in the Supporting Information). Thus, the decline in halo definition, despite the fact that there was an increase in Ste2p protein, indicates that Bpa was incorporated into these receptors but the function was compromised.

For the mutant at position Y193, no halos were formed in the absence of Bpa, suggesting that no functional Ste2p was expressed (Figure 2O), although a low-level full-length protein was detected by immunoblot analysis with the C-terminal antibody (Figure 3A). In contrast, in the presence of Bpa, dose-dependent fuzzy halos were formed, indicating that Bpa was inserted in response to the TAG codon, resulting in a functional protein (Figure 2P). In parallel with this increase in Ste2p function, there was a concomitant increase in protein expression (Figure 3A) for cells grown in the presence of Bpa. The $G188^{TAG}$ mutant had a similar phenotype (parts M and N of Figure 2) but with less defined halos observed only at the highest amount of pheromone and only in the presence of Bpa. The poor definition of the halos suggested that Bpa was not well-tolerated at this position. For the G188 receptor, there was no detectable protein produced in the absence of Bpa and a weak but clearly discernible band was detected in membranes obtained from cells grown in the presence of Bpa (Figure 3A). Finally, the $Y266^{TAG}$ receptor was not functional in either the presence or absence of Bpa (parts S and T of Figure 2), and immunoblot analysis (see Figure S1 in the Supporting Information) indicated that this receptor was not expressed under either condition.

C-Terminally truncated forms of the receptor can be seen for the $G188^{TAG}$ and $Y193^{TAG}$ receptors using antibody to the N terminus (Figure 3B). Three major bands were detected at 23, 21, and 18 kDa for the $G188^{TAG}$ and at 24, 22, and 19 kDa for the $Y193^{TAG}$ receptor. These bands corresponded to the expected size of the nonglycosylated receptor and its two major glycosylated states truncated at the inserted stop codons (*37*). The origin of the bands of higher molecular weight (between 22 and 50 kD) in the $G188^{TAG}$ and $Y193^{TAG}$ lanes (Figure 3B) was not investigated, although these bands are not related to the absence or presence of Bpa. Truncated forms of the $F55^{TAG}$ receptor were not detected with the N-terminal antibody (Figure 3B) on this immunoblot because the predicted receptor fragment (~6 kD) would not have been retained on the gel. Note that the full-length Ste2p band intensity (52 kD) for the WT strain is less intense as detected by the N-terminal antibody (Figure 3B) than that of the same membranes probed with the FLAG antibody (Figure 3A).

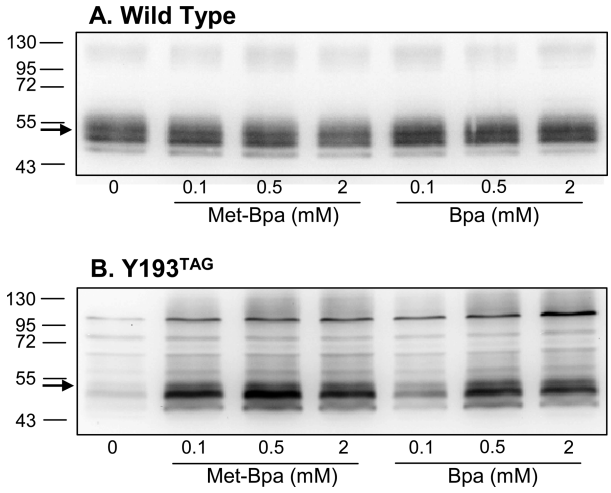

FIGURE 4: Use of a peptidyl form of Bpa. FLAG immunoblot analysis of membranes from cells expressing either WT (A) or Y193^{TAG} (B) Ste2p receptors grown in the presence of the dipeptide Met−Bpa or free Bpa at the concentrations indicated. Protein loads were 2 and 30 μg for the WT and Y193 mutant, respectively.

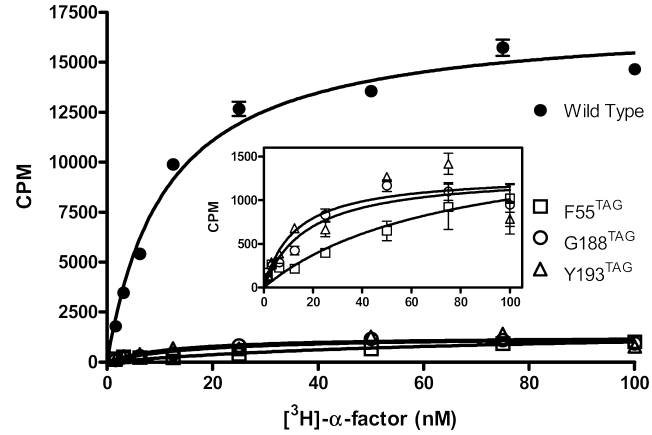

FIGURE 5: Whole cell saturation binding assay of [^{3}H]α factor to WT Ste2p and Ste2p−TAG mutant receptors. Cells expressing the WT receptor and the indicated Bpa mutants were grown in the presence of 2 mM Bpa. (Inset) Saturation binding assay for TAG mutant receptors plotted on an expanded scale. The data represent specific binding to cells as determined by subtracting the binding to an isogenic strain lacking the receptor from binding to cells containing WT or mutant Ste2p.

In our hands, the FLAG antibody always yields a stronger signal than the N-terminal antibody. This provides an explanation for the observation that, although full-length Bpa-containing proteins can be detected with the FLAG antibody, they are barely discernible by the N-terminal antibody. Expression of the full-length F55^{TAG} Bpa-containing receptor was enhanced in response to an increased Bpa concentration in the growth medium (Figure 3C). At a low concentration (0.2 mM Bpa), no increase over control (no Bpa) was observed, while at higher concentrations (2 mM), the signal corresponding to Ste2p on the immunoblot was enhanced, increasing by 2.6-fold over the control as measured by band intensity. Similar results were obtained for the G188^{TAG} and Y193^{TAG} mutants (data not shown), suggesting that entry of Bpa into the cell is one factor determining the efficiency of Bpa insertion into the protein.

Effect of Bpa-Containing Peptides on the Expression of Ste2p. The data for the F55^{TAG}, G188 ^{TAG}, and Y193 ^{TAG} receptors described above suggests that the expression of Bpa-containing Ste2p could be enhanced if Bpa were more efficiently delivered into the cell. It is known that small peptides enter yeast cells intact through the di/tripeptide transporter Ptr2p (*38, 39*), and upon entry into the cell, these peptides are hydrolyzed to free amino acids. In an attempt to increase the delivery of Bpa into the cell, a Bpa-containing dipeptide (Met−Bpa) was synthesized. Methionyl di- and tripeptides are excellent substrates for the yeast peptide transport system (*40*). Cells expressing the F55^{TAG}, G188^{TAG}, and Y193^{TAG} receptor constructs were grown in the presence and absence of Met−Bpa, and membranes were isolated and probed with the C-terminal FLAG antibody to detect full-length protein. The expression of full-length WT Ste2p was not affected by the presence of Met−Bpa, confirming that this compound did not interfere with normal Ste2p expression at the concentrations indicated (Figure 4A). The Y193^{TAG} mutant expressed minimal FLAG-reactive Ste2p in the absence of Bpa, but in the presence of Met−Bpa (Figure 4B), full-length protein was detected. In comparison to the expression of Y193^{TAG} grown in the presence of 0.1 mM Met−Bpa to that grown in the presence of 0.1 mM Bpa (Figure 4B), expression was greater for the dipeptide (3.1-fold increase). At higher concentrations (0.5 and 2 mM), growth on Met−Bpa and Bpa yielded similar levels of expression (1.4-fold increase at each concentration). Similar results were obtained for the F55^{TAG} and Y193^{TAG} mutants. Growth on dipeptide at 0.1 mM resulted in 2.4- and 2.0-fold increases in expression compared to growth on 0.1 mM Bpa for the F55^{TAG} and Y193^{TAG} mutants, respectively. At higher concentrations of peptide (0.5 mM and 2 mM), growth on Met−Bpa did not increase Ste2p expression when compared to the same concentration of Bpa.

Cell-Surface Expression of Bpa Mutants Determined by Saturation Binding Assay. To characterize the cell-surface expression and binding affinity of the Ste2p Bpa mutants, DK102 cells expressing the F55^{TAG}, G188^{TAG}, and Y193^{TAG} receptors were used in whole-cell binding assays. Binding of radiolabeled α factor was detected for cells expressing the WT receptor when cultured in either the presence or absence of Bpa. In experiments using the WT receptor, Bpa did not have any significant effect on the B_{max} and binding affinity (B_{max} = 152 470 ± 6900 receptors per cell, K_D = 9.7 ± 1.4 nM) when compared to cells cultured in the absence of Bpa (B_{max} = 158 100 ± 6500 receptors per cell, K_d = 11.0 ± 1.0 nM). For the mutant receptors, binding was not detected for cells grown in the absence of Bpa (data not shown). However, for cells grown in the presence of Bpa, saturable binding was observed (Figure 5). The B_{max} values for the mutants were reduced by 10−20-fold compared to the WT receptor (Figure 5), which is consistent with the low level of expression observed in the immunoblots (Figure 3). The binding affinity (K_d) of the Y193^{TAG} and G188^{TAG} mutants (12.8 ± 4.9 and 17.5 ± 5.7 nM, respectively) were similar to that for the WT receptor, indicating that substitution of Bpa for the endogenous amino acid at these positions did not affect the interaction of the ligand with the receptor. In contrast, for the F55^{TAG} mutant, the binding affinity was reduced to 78.9 ± 46.1 nM, indicating that the substitution of Bpa for phenylalanine at this position affected ligand binding, which correlates to the relative low signaling activity of this receptor.

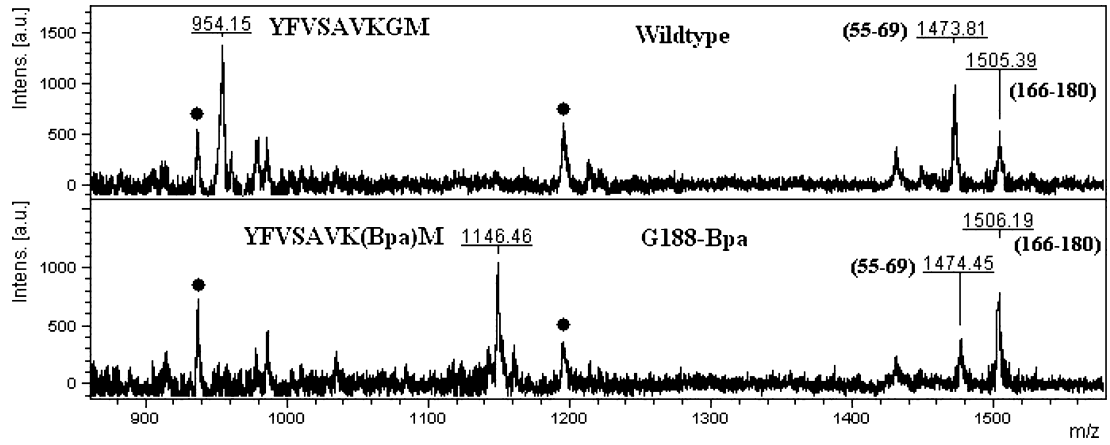

FIGURE 6: MALDI−TOF analysis of CNBr cleavage fragments of WT and G188^{TAG} receptors. A peak (954.15 Da) corresponding to residues 181−189 (YFVSAVKGM) in the WT receptor was not present in the G188^{TAG} receptor. The masses for the CNBr fragments indicated that the carboxyl-terminal methionine is converted to homoserine lactone. A new peak (1146.46 Da) corresponding to 181−189 with Bpa at position 188 was detected. Other peaks corresponding to residues 55−69 and 166−180 were observed for both WT and G188^{TAG} receptors. Peaks labeled with a black dot do not correspond to any to the theoretical masses for CNBr cleavage fragments of Ste2p.

Table 1: Summary of MALDI−TOF Results for WT and the G188^{TAG} Mutant Grown in the Presence of Bpa[a]

WT		G188^{TAG} (+Bpa)		corresponding CNBr fragment (residues of Ste2p)
theoretical mass	observed mass	theoretical mass	observed mass	
953.11	954.15	**1147.34**	**1146.46**	181−189
1474.82	1473.81	1474.82	1474.45	55−69
1506.77	1505.39	1506.77	1506.19	166−180

[a] Note that incorporation of Bpa into the mutant protein increases the theoretical mass (bold) for the CNBr fragment corresponding to residues 181−189 [YFVSAVK(Bpa)M], which is also reflected in the observed mass (bold) for that same fragment. The theoretical masses for the CNBr fragments presumes that the carboxyl-terminal methionine is converted to homoserine lactone.

MALDI−TOF Indicates that Bpa Is Incorporated into Ste2p at Position G188. To confirm the incorporation of Bpa into Ste2p, MALDI−TOF mass spectrometric analysis of purified WT and G188^{TAG} receptors was performed. The G188^{TAG} receptor was chosen for this analysis based on nearby naturally occurring methionine sites in Ste2p that would yield a small peptide (residues 181−189) upon CNBr cleavage to facilitate mass spectroscopy. The substitution of glycine (75.07 Da) for Bpa (269.30 Da) at position 188 should result in a mass shift of 194 Da in the 188Bpa-containing peptide. To estimate purity and concentration of the samples prior to CNBr cleavage and MALDI−TOF analysis, Coomassie Blue and silver staining of SDS−PAGE gels was performed on the purified G188^{TAG} and WT receptors. In the Coomassie-stained gel, only two bands, corresponding in molecular weight to the glycosylated and nonglycosylated forms of Ste2p (50−52 kDa), were observed. Using the more sensitive silver-staining technique on the same sample, the same two bands were detected, along with some very faint bands not corresponding to Ste2p. Immunoblot analysis confirmed that the two major bands were Ste2p (data not shown). After CNBr cleavage of WT and G188^{TAG} receptors, we carried out a MALDI−TOF analysis of the peptide fragments generated, focusing on the molecular-weight region corresponding to the peptide with the putative Gly to Bpa substitution. Our analysis of the CNBr-cleaved WT and G188^{TAG} receptors (Figure 6) revealed a mass shift from 954.154 Da in WT to 1146.46 Da in G188^{TAG}, corresponding to a 192 Da increase (Table 1). Other peaks of identical mass corresponding to CNBr fragments of Ste2p were observed in both the WT (1473.81 and 1505.39 Da) and G188Bpa (1474.45 and 1506.19 Da) receptors. Some peaks not corresponding to any of the theoretical masses of Ste2p CNBr fragments were also observed and are likely due to the degradation of Ste2p, which occurred during the overnight incubation at 37 °C during CNBr cleavage, or the presence of other peptides in the preparation.

Bpa at Positions F55 and Y193 Can Cross-link to the α-Factor Ligand. To determine if Bpa incorporated into Ste2p could be used to cross-link to and thus capture the α-pheromone, cells expressing the WT, F55^{TAG}, G188^{TAG}, and Y193^{TAG} receptors as well as cells lacking Ste2p as a control were grown in the presence of Bpa. Membranes were prepared and incubated with or without [K^7(biotinylamidocaproate)]α factor, an α factor analogue that contains biotin on the position seven lysine of the pheromone and binds to the receptor with high affinity (*27*). Cross-linking was also evaluated in the presence or absence of 100-fold excess nonbiotinylated α factor. After incubation, the membranes were exposed to UV light to activate capture of pheromone to the Bpa-labeled receptor. The membranes were fractionated by SDS−PAGE, blotted, and then probed with FLAG antibody to detect Ste2p (Figure 7A) or Neutravidin−HRP to detect biotinylated ligand (Figure 7B). WT receptor was expressed at levels higher than any of the mutant receptors (Figure 7A) as seen in previous experiments (Figure 3). When the immunoblots were probed with Neutravidin−HRP (Figure 7B), a distinct band was detected at the size expected for Ste2p in the F55^{TAG} and Y193^{TAG} receptors, indicating that the biotinylated α factor was cross-linked. Despite the fact that at least 20 times as much WT Ste2p was expressed when compared to the F55^{TAG} or Y193^{TAG} receptors (Figure 7A), labeling as detected with Neutravidin−HRP was significantly higher for the mutants (Figure 7B). Moreover, labeling of the F55^{TAG} and Y193^{TAG} Bpa-containing receptors was reduced in the presence of excess nonbiotinylated α factor (Figure 7B) by 83 and 64%, respectively, as determined by quantitation of the band density. Similar results were observed in three independent

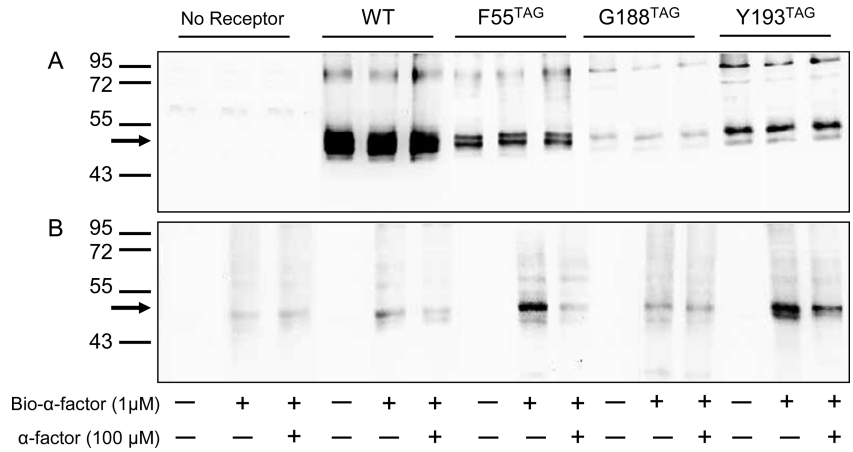

FIGURE 7: Cross-linking of biotinylated α factor into Bpa-containing receptors. Membranes prepared from cells expressing the WT, F55^{TAG}, G188^{TAG}, and Y193^{TAG} receptors grown in the presence of Bpa were incubated with [K^{7}(biotinylamidocaproate), Nle^{12}]α factor (1 μM) in the presence (+) or absence (−) of nonbiotinylated α factor (100 μM). Membranes were also prepared from cells lacking the receptor as a negative control. (A) After photoactivation, membranes (2 μg for WT and 25 μg for mutants and the negative control) were immublotted and probed with FLAG antibody to detect Ste2p. (B) Membranes (25 μg for each lane) were immunoblotted and probed with Neutravidin−HRP to detect biotinylated ligand. The arrow in both panels indicates the expected size for Ste2p.

replicates of this experiment. For membranes prepared from cells lacking the receptor, as well as for the WT and the G188^{TAG} receptors, a faint band at approximately 50 kD was observed (Figure 7B) that was dependent upon incubation of the membranes with biotinylated pheromone. The signal was not affected by the presence of nonbiotinylated pheromone. Thus, this signal is the result of nonspecific association of the biotinylated pheromone with the membranes in a Ste2p-independent manner.

DISCUSSION

Many experiments have been performed with a variety of methodologies to provide a wealth of knowledge concerning GPCR structure and function. However, an understanding of the mechanism of activation is still in its infancy. The gold standard would be to obtain crystal structures of the GPCR in both the resting and active state. Thus far, bovine rhodopsin and the human β-adrenergic receptor are the only GPCRs to have had their crystal structure solved (*41–43*). For rhodopsin, the receptor was crystallized in the inactive state, although recent reports of an active-state crystal at moderate to low resolution (2.7−5.5 Å) have been published (*44*). Crystallization of the β-adrenergic receptor required expression as a fusion protein (*43*) or interaction with an inverse agonist (*42*) to stabilize receptor conformation. The use of unnatural amino acid replacement provides an alternate method for examining the receptor structure as well as exploring the changes that occur in GPCRs upon receptor activation.

In an earlier study, a fluorescent amino acid was incorporated into a GPCR, the neurokinin-2 receptor, using heterologous expression in *Xenopus* oocytes (*12*). In this paper, we report the first evidence that an unnatural amino acid can be incorporated into the GPCR Ste2p expressed in its native environment in the yeast cell and still retain function. The incorporation was verified by mass spectrometry, and our studies indicate that, once incorporated, the photoactivatable Bpa in Ste2p can be used to covalently link the α-factor ligand to the receptor. Although there are many examples in the literature of photoactivated cross-linking of ligand to receptor, in all of those cases, the ligand contained the photoactivatable moiety. To our knowledge, our study represents the first example of cross-linking of ligand to a GPCR in which the receptor contained the photoactivatable group used to "capture" the ligand.

We demonstrated that incorporation of Bpa into both the F55 (located in TM1) and Y193 (located in EL2) receptors resulted in capture of biotinylated pheromone. Photochemical capture of the ligand could be largely inhibited by the presence of excess nonbiotinylated pheromone. While F55 is positioned in the binding pocket of the receptor and was expected to interact with the pheromone (*27*), the ability of Y193 to capture the ligand suggests a possible role for EL2 in ligand binding. Previous studies using domain swapping indicated that EL2 was not a determinant of ligand specificity of the *S. cerevisiae* and *S. kluyveri* α-factor receptors (*45*). Thus, our observation of a putative contact between the EL2 region of Ste2p and α factor indicates the need for further study of EL2−α-factor interactions. Because of the low level of protein expression, it was unclear whether the G188 receptor was functional with respect to ligand capture, despite the fact that this receptor was determined to contain Bpa by mass spectrometry analysis (Figure 6). Saturation binding analysis (Figure 5) indicated that the G188^{TAG} receptor was expressed on the cell surface at levels comparable to that of the F55^{TAG} receptor, in which we were able to detect cross-linking of the receptor to the pheromone. In this light, despite the low level of total protein expression observed on the immunoblot, it is likely that there is enough of the G188^{TAG} receptor on the cell surface to allow for cross-linking if the pheromone has access to the Bpa residue. Previously published data using cysteine scanning of Ste2p indicated that G188 is between 25 and 50% exposed on the basis of the reaction with sulfhydryl reagents (*46*); thus, it is accessible to the solvent. If the pheromone was interacting nonspecifically with the receptor or if residue G188 was involved in ligand binding, then Bpa at this position should have been able to capture ligand.

In addition to ligand capture experiments, we can envision other applications of this mutagenesis methodology, such as studying changes in domain−domain interactions upon ligand binding, which will shed light on the receptor

activation mechanism. An advantage of using the orthologous tRNA/aminoacyl-tRNA synthetase pair to incorporate Bpa into Ste2p in the yeast cell is that the GPCR remains in its native environment and is thus able to interact with downstream effector molecules, such as the heterotrimeric G-proteins, thus making assessment of both ligand binding and signal transduction possible.

Although the use of unnatural amino acids has the potential to answer very important questions in GPCR biology, there are still obstacles to overcome. In the experiments presented in this paper, eight mutant *STE2* constructs ($F55^{TAG}$, $S107^{TAG}$, $G115^{TAG}$, $V127^{TAG}$, $G188^{TAG}$, $Y193^{TAG}$, $F204^{TAG}$, and $Y266^{TAG}$) were created with the amber TAG stop codon engineered at different sites within the open-reading frame. Theoretically, when the cells expressing these mutant receptors were grown in the presence of Bpa, the unnatural amino acid would be incorporated into the protein by the cognate tRNA/aminoacyl-tRNA synthetase also expressed in the cell. In the absence of Bpa, it was expected that the TAG codon would result in termination of protein synthesis, resulting in a truncated protein. For the mutants $G188^{TAG}$ and $Y193^{TAG}$, this was the case; in the presence of Bpa, cells responded to pheromone (Figure 2) and full-length receptor was detected by immunoblot analysis (Figure 3) and both bound ligand (Figure 5) with near WT affinity. However, mutants with TAG amber stops at positions F55, S107, G115, V127, and F204 exhibited variable amounts of inefficient stop codon recognition or read-through expression, as evidence by the halo assay, which in the context of the present experiments results in the production of full-length protein even in the absence of Bpa.

The efficiency with which nonsense codons, such as the UAG amber stop in mRNA, are suppressed can be affected by the context of the adjacent codons and poses a challenge (*47*). In *S. cerevisiae*, nucleotides within six bases flanking the stop codon can influence the efficiency of termination (*48*) and it has been determined that "backup" or tandem stop codons can be found three codons downstream of the authentic stop for some genes (*49*). In the case of read-through expression, noncognate tRNA species can misread the stop codon and insert an inappropriate amino acid. In the case of the UAG codon in yeast mRNA, the $tRNA^{Gln}_{GUC}$ can insert glutamine at the stop codon when it is present in a context unfavorable for release-factor recognition (*50*). In another tRNA/aminoacyl-tRNA synthetase system evolved to incorporate the fluorescent amino acid dansyl-alanine in response to the amber stop codon, read-through expression in the absence of dansyl-alanine was eliminated by mutation of the aminoacyl-tRNA synthetase to increase selectivity of the enzyme for dansyl-alanine (*18*). Thus, to optimize the system for insertion of Bpa into Ste2p, genetic manipulations in protein translation machinery (i.e., ribosomes, tRNAs, etc.) might be warranted.

Interestingly, for the mutants $F55^{TAG}$ and $F204^{TAG}$, protein function but not expression was eliminated when the cells were grown in the presence of Bpa (Figures 2 and 3 and Figure S1 in the Supporting Information). This indicates that the unnatural amino acid was incorporated into the protein but did not result in a signal-transducing receptor at the cell surface. The presence of mutant Ste2p or other GPCRs that are expressed and bind ligand but do not signal has been extensively reported (*3*). Indeed, binding without signaling is characteristic of antagonists, and mutation of GPCRs to preferentially recognize antagonists has been documented (*32, 33*).

Incorporation of Bpa into the various TAG mutant receptors was not 100% efficient, as can be seen by the presence of truncated receptors on immunoblots (Figure 3B), which could potentially interfere with the function of a full-length receptor. However, previous studies showed that overexpression of 14 different truncated Ste2p receptors failed to produce any significant alteration in Ste2p function (*51*). Thus, the loss of signaling function ($F55^{TAG}$ and $F204^{TAG}$) in the halo assays that we observed likely results from intolerance of Bpa at the positions into which it was inserted rather than to the interference by truncated receptors.

Using the orthologous tRNA/aminoacyl-tRNA synthetase pairs to incorporate Bpa at the engineered amber stop codon requires the presence of Bpa in the cytoplasm at sufficient concentrations to allow for effective charging of the tRNA. We have used a novel means to supply Bpa to yeast cells via the di/tripeptide transport system that is common to many eukaryotes (*52, 53*). In yeast, di/tripeptides as well as oligopeptides (tetra/pentapeptides) are not cleaved outside the cell but are transported intact across the plasma membrane (*54, 55*). Expression of the $Y193^{TAG}$ receptor as well as the $F55^{TAG}$ and $G188^{TAG}$ receptors was noticeably greater when the cells were grown in the presence of Met−Bpa at 0.1 mM when compared to Bpa (Figure 4). Because peptide transporters are ubiquitous in living cells (*53*), this method might be used to enhance the delivery of unnatural amino acids and extend this approach to amino acids that cannot enter cells through amino acid permeases.

In summary, in this paper, we provide evidence that the unnatural photoactivatable amino acid *p*-benzoyl-L-phenylalanine can be incorporated into a GPCR in its native cell. The $Y193^{TAG}$ mutant receptor responded to α factor and bound this ligand with nanomolar affinity. Other mutant receptors examined were expressed at the membrane at levels from 5 to 10% of the WT receptor under identical conditions. Mass spectrometry proved that Bpa was incorporated at the expected position for the $G188^{TAG}$ receptor. Expression could be increased when the unnatural amino acid was delivered as a dipeptide via the peptide transport system of *S. cerevisiae*. The enhancement of Bpa delivery as a dipeptide was most effective at low (0.1−0.5 mM) concentrations; at 2 mM, both dipeptide and Bpa were equally effective. Two Bpa-containing receptors were able to capture ligand after photoactivation, providing evidence for the utility of such labeled GPCRs to determine ligand−receptor interactions. These results set the stage for the use of unnatural amino acids technology in exploring the structure and function of integral membrane proteins in their native environment.

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SUPPORTING INFORMATION AVAILABLE

Immunoblots of membranes isolated from cells expressing Ste2p mutants with the TAG codon inserted at specific

residues (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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