Cross-linking Strategies to Study Peptide Ligand–Receptor Interactions

ARTICLE *in* METHODS IN ENZYMOLOGY · APRIL 2015

Impact Factor: 2.09 · DOI: 10.1016/bs.mie.2014.12.001 · Source: PubMed

2 AUTHORS:

Jeffrey M Becker

University of Tennessee

290 PUBLICATIONS **6,814** CITATIONS

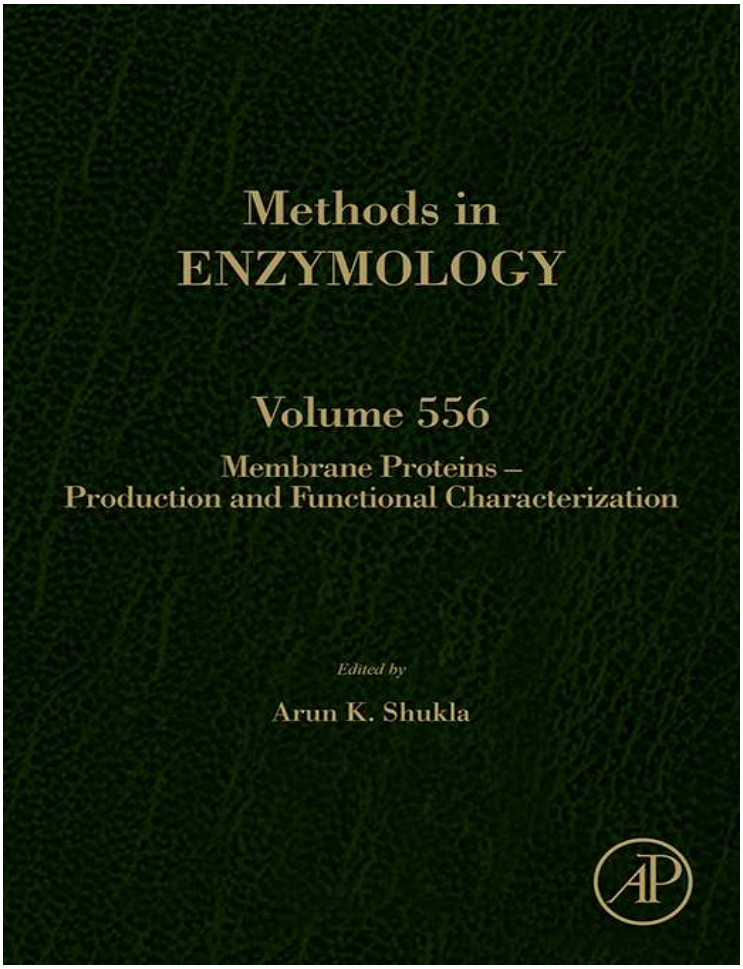
SEE PROFILE

Fred Naider

City University of New York - College of Stat…

287 PUBLICATIONS **6,357** CITATIONS

SEE PROFILE



From Jeffrey M. Becker and Fred Naider, Cross-linking Strategies to Study Peptide Ligand–Receptor Interactions. In: Arun K. Shukla, editor, *Methods in Enzymology, Vol. 556*, Burlington: Academic Press, 2015, pp. 527-547.
ISBN: 978-0-12-801521-6

CHAPTER TWENTY-FOUR

Cross-linking Strategies to Study Peptide Ligand–Receptor Interactions

Jeffrey M. Becker[*,1], **Fred Naider**[†]
[*]Microbiology Department, University of Tennessee
[†]Chemistry Department, College of Staten Island, City University of New York
[1]Corresponding author: e-mail address: jbecker@utk.edu

Contents

1. Introduction 528
2. Photochemical Cross-linking 529
   2.1 Synthesis of photochemically active peptides with biotin tag: Case example [Bpa[1],Lys[7](biotinylamidocaproate),Nle[12]]α-factor 530
   2.2 Synthesis of α-Fmoc[Bpa[1],Nle[12]]α-factor 530
   2.3 Synthesis of [Bpa[1], K(BiotinACA)Nle[12]]α-factor 532
   2.4 Cross-linking conditions for Bpa-labeled peptides 533
   2.5 Cross-linking of [Bpa[1], K(BiotinACA)Nle[12]]α-factor into Ste2p 533
3. Chemical Cross-linking 534
   3.1 Synthesis of DOPA-biotinylated peptides 535
   3.2 Synthesis of [DOPA[1],Lys[7](BioACA),Nle[12]]α-factor 535
   3.3 Cross-linking conditions for DOPA-labeled peptides 535
4. Cross-Linking by Unnatural Amino Acid Replacement into the Receptor 537
   4.1 Ligand capture by unnatural amino acid replacement in Ste2p 538
   4.2 Incorporation of the unnatural amino acid Bpa into Ste2p 538
5. Identifying the Cross-link Site 539
   5.1 Isolating the tagged receptor–ligand complex 539
   5.2 Fragmenting cross-linked peptide–protein complex 540
   5.3 Isolating cross-linked fragment 541
   5.4 Mass spectrometric analysis of the cross-linked fragment 541
6. Concluding Remarks 543
Acknowledgments 544
References 544

Abstract

Experiments are described that allowed cross-linking of analogs of a 13-amino acid peptide into the binding site of a model G protein-coupled receptor. Syntheses of peptide analogs that were used for photochemical or chemical cross-linking were carried out using solid-phase peptide synthesis. Chemical cross-linking utilized 3,4-dihydroxy-L-

Methods in Enzymology, Volume 556
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2014.12.001

phenylalanine-incorporated peptides and subsequent periodate-mediated activation, whereas photochemical cross-linking was mediated by *p*-benzoyl-L-phenylalanine (Bpa)-labeled peptides and UV-initiated activation. Mass spectrometry was employed to locate the site(s) in the receptor that formed the cross-links to the ligand. We also describe a method called unnatural amino acid replacement that allowed capture of a peptide ligand into the receptor. In this method, the receptor was genetically modified by replacement of a natural amino acid with Bpa. The modified receptor was UV-irradiated to capture the ligand. The approaches described are applicable to other peptide-binding proteins and can reveal the ligand-binding site in atomic detail.

1. INTRODUCTION

An understanding of the function of a membrane receptor starts with determination of its structure and the nature of its ligand-binding site. Impressive advances have been made in the last decade in membrane protein expression and purification resulting in the use of X-ray crystallography on membrane protein crystals of G protein–coupled receptors (GPCRs) to elucidate overall structure, and in some cases, an atomic-level image of the ligand-binding site (Bortolato et al., 2014; Cherezov et al., 2007; Chien et al., 2010; Chung et al., 2011; Hanson & Stevens, 2009; Jaakola et al., 2008; Katritch, Cherezov, & Stevens, 2012; Rasmussen et al., 2011; Scheerer et al., 2009; Warne et al., 2008, 2011; Wu et al., 2010, 2014).

Approximately, 360 genes encode members of a human membrane protein family composed of nonolfactory GPCRs (Fredriksson, Lagerstrom, Lundin, & Schioth, 2003). The study of the GPCR family is especially compelling in light of the fact that a large proportion of drugs for human medicine modulate the activity of GPCRs (Garland, 2013; Lappano & Maggiolini, 2011). However, only a handful of high-resolution structures have been solved for nonolfactory GPCRs; demand remains for methodologies to uncover the nature of the interaction of GPCRs with ligands including agonists/antagonists and other allosteric effector molecules. The goals of such methodologies include elucidation of the atom-to-atom interactions between ligand and receptor. Such knowledge informs drug design and the development of mechanisms for signal transduction via conformational changes in the receptor that result in gene regulation and metabolic remodeling of cellular activity.

Our lab has used the yeast GPCR (Ste2p) interacting with the tridecapeptide α-mating factor to study peptide ligand–receptor interactions (Akal-Strader, Khare, Xu, Naider, & Becker, 2002; Hauser, Kauffman, Lee,

Naider, & Becker, 2007; Huang et al., 2008; Kim, Lee, Naider, & Becker, 2009; Kim et al., 2012; Raths, Naider, & Becker, 1988; Son, Sargsyan, Hurst, Naider, & Becker, 2005; Son, Sargsyan, Naider, & Becker, 2004; Umanah et al., 2010; Umanah, Huang, Maccarone, Naider, & Becker, 2011; Umanah, Son, Ding, Naider, & Becker, 2009; Xue, Eriotou-Bargiota, Miller, Becker, & Naider, 1989). This yeast GPCR model system was among the first to uncover the amino acid sequence of a GPCR (Burkholder & Hartwell, 1985; Nakayama, Miyajima, & Arai, 1985), G protein coupling, downstream intracellular signaling events involving a protein kinase cascade, and intracellular regulatory reactions (Dohlman, Thorner, Caron, & Lefkowitz, 1991; Wang & Dohlman, 2004; Wu, Hooks, Harden, & Dohlman, 2004). Yeast genetics and molecular biology provided powerful tools that facilitated these discoveries.

In this review, we highlight experiments that allowed the cross-linking of α-factor analogs into the binding site of Ste2p. For these studies, α-factor analogs were synthesized and photochemically (Henry et al., 2002; Son et al., 2004, 2005) or chemically (Umanah et al., 2009, 2010) cross-linked into Ste2p. Mass spectrometry was used to locate the site(s) in the receptor that formed the cross-link to the ligand. We have also used a method called unnatural amino acid replacement (UAAR) pioneered for yeast in the laboratory of Prof. Peter Shultz (Chin et al., 2003; Huang et al., 2008). In this method, the photoactivatable, unnatural amino acid *p*-benzoyl-L-phenylalanine (Bpa) was inserted into Ste2p by genetic engineering, and the modified receptor was UV-irradiated to capture the ligand (Huang et al., 2008). All these approaches are applicable to other receptors and ligands in order to reveal the ligand-binding site in atomic detail.

2. PHOTOCHEMICAL CROSS-LINKING

Cross-linking of peptide ligands into proteins may be accomplished by chemical or photochemical approaches. The advantage of the latter method is that temporal control of the reaction is possible in that the active species is created only upon irradiation. The disadvantage is that the free radical associated with photoactivation can react with almost any C–H bond in the protein. Furthermore, depending on the lifetime of the active state it is possible that reaction can occur over a long distance. We and others have used photoactivation of peptides containing Bpa extensively to identify contact points in enzymes (Dorman & Prestwich, 1994; Sumranjit & Chung, 2013), and receptors (Fillion et al., 2013; Grunbeck, Huber, Sachdev, & Sakmar,

2011; Grunbeck, Huber, & Sakmar, 2013; Rihakova et al., 2002; Wittelsberger, Mierke, & Rosenblatt, 2008). Bpa is commercially available in Fmoc and Boc forms needed for solid-phase peptide synthesis, and it is a stable derivative not readily degraded by indoor light. Peptides containing Bpa are stable for years when stored in the freezer. The steps delineated below may be followed for most peptide sequences to produce a Bpa-containing peptide to use for cross-linking into a membrane protein.

2.1. Synthesis of photochemically active peptides with biotin tag: Case example [Bpa^{1},Lys^{7}(biotinylamidocaproate), Nle^{12}]α-factor

Alpha-factor is a tridecapeptide [Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr] mating factor that activates Ste2p, a GPCR in yeast. The synthesis of this peptide and its Bpa analogs is readily accomplished by automated solid-phase peptide synthesis using Fmoc protection and HBTU/HOBt activation. We normally use the Applied Biosystems 433A peptide synthesizer on a 0.1 mmol scale. For the α-factor derivatives, this results in a yield after purification of α-Fmoc-protected peptide of 50–100 mg with 90–95% homogeneity. This intermediate is then biotinylated in solution. The following protocol was published in Son et al. (2004) and was slightly modified for this chapter.

2.2. Synthesis of α-Fmoc[Bpa^{1},Nle^{12}]α-factor

Notes: Reactions not done in the synthesizer are protected from light. All automated procedures may be done manually using the same coupling, deprotection, and wash steps.

(1) All N-α-Fmoc amino acid derivatives and N-α-Fmoc-Tyr(OtBu)-Wang resin are commercially available. We purchase these from Advanced Chemtech (CreoSalus, Louisville, KY) and from Novabiochem (EMD Chemicals, Gibbstown, NJ). We pack the cartridges used in synthesis by hand. Prepacked cartridges are available from Applied Biosystems (Foster City, CA).

(2) N-α-Fmoc-Tyr(OBu^{t})-Wang resin (0.7 mmol substitution/gram resin) is placed into the reaction vessel.

(3) Reaction vessel is placed into the synthesizer.

(4) 0.1 mmol FastMoc chemistry is used for elongation with HBTU/HOBt single-step coupling.

(5) The coupling time is 30 min with a total cycle time of 1.5 h for one amino acid elongation.

(6) Single coupling steps are employed with 10-fold molar excess of amino acid.

(7) Fmoc removal is catalyzed by 20% piperidine in *N*-methyl-pyrrolidone treatment for 2 min with the cleavage reaction repeated three to five times.

(8) After the insertion of N-α-Fmoc-Bpa, the Fmoc-cleavage cycle is omitted and the resulting N-α-Fmoc-protected-peptidyl resin thoroughly washed with 1-methyl-2-pyrrolidone and dichloromethane and dried in a vacuum for 2 h.

(9) The resin is transferred to 20-mL vial containing a stir bar and a screw top cap.

(10) Cleavage-deprotection cocktail [TFA (10 mL), crystalline phenol (0.75 g), thioanisole (0.5 mL), and water (0.5 mL)] is added and the reaction stirred at room temperature for 1.5 h. [Note: Ethanedithiol often used in cleavage cocktails should be omitted because it leads to a thioketal derivative of the diphenylketone moiety of Bpa.]

(11) The reaction is filtered through sintered class to remove the resin, the resin washed two times with 5 mL of trifluoroacetic acid (TFA) and all washes combined.

(12) The combined peptide solutions are transferred to a 100-mL round-bottomed flask and most of the solvent removed under vacuum in a rotary evaporator at a temperature below 30 °C.

(13) Cold diethyl ether (30–40 mL) is added to the peptide slurry to precipitate the peptide.

(14) All of the content of the round bottom flack is transferred into 50-mL centrifuge tube. The crude peptide precipitate is recovered by centrifugation in a 50-mL centrifuge tube, washed three times with ether applying sonication and centrifugation every time to isolate peptide. Precipitated crude peptide is dried in vacuum overnight.

(15) The yield of the crude peptide is usually about 80% (∼150–200 mg).

(16) The crude peptide (∼30–40 mg) is dissolved in ∼4 mL of aqueous acetonitrile (20%) containing 0.1% TFA.

(17) The solution is loaded onto a semipreparative Waters DeltaPak column C18 (19 mm × 300 mm) and eluted with a water/acetonitrile linear gradient containing 0.1% TFA (10–70% acetonitrile over 80 min at a flow rate of 5 mL/min).

(18) Peak detection is at 280 n*M*, up to 10 fractions are collected during major peak elution.

(19) Collected fractions are analyzed at 220 n*M* on an analytical Zorbax Eclipse XDB-C8 column (4.6 mm × 150 mm).

(20) Purity of the final product is assessed in acetonitrile/water/0.1% TFA system and is usually >95% with overall yield of HPLC purification up to 40–50%.

(21) Molecular weight of the peptide is determined by ESI-MS.

(22) The Fmoc-[Bpa^1,Nle^{12}]α-factor can now be biotinylated at the ε-amine of Lys.

2.3. Synthesis of [Bpa^1, K(BiotinACA)Nle^{12}]α-factor

(1) Fmoc-BpaHWLQLKPGQPNleY (27 mg, 12.4 μmol) is dissolved in DMF (2 mL) and 50 m*M* $Na_2B_4O_7$(1.5 mL) at 4 °C.

(2) A solution of biotinamidohexanoic acid *N*-hydroxysuccinimide ester (Sigma #B2643) (BiotinACA-OSu) (8.5 mg, 18.6 μmol) in 0.5 mL DMF is added and the resulting mixture stirred at 4 °C for 1 h. [Note: This reaction may be done in two steps with purification of the Fmoc biotinylated intermediate. We have not found this to improve product quality and the extra HPLC step leads to loss of material.]

(3) The reaction is monitored by HPLC, and when judged complete based on loss of starting material 160 μL of piperidine added to remove the Fmoc protection group.

(4) After 1 h the reaction is quenched by addition of 12 N HCl (0.2 mL), the solution filtered and the entire filtrate (~4.5 mL) injected into preparative HPLC Waters C-18 Bondapak column 300 × 19 mm via two injections using 20–60% acetonitrile (+0.1% TFA) gradient over 60 min.

(5) This step is a prepurification to remove solvents and reagents.

(6) The impure peptide fraction is collected and lyophilized.

(7) Crude peptide (8 mg) is dissolved in 2 mL 20% acetonitrile (+0.1% TFA) and purified using a Waters C-18 DeltaPak column 300 × 19 mm and 10–80% acetonitrile(+0.1% TFA) gradient over 90 min.

(8) Purity of peptide is assessed by analytical HPLC using Zorbax Eclipse XDB-C8 4.6 × 150 mm column and 10–90% acetonitrile (+0.1% TFA) gradient.

(9) Pure fractions (>99%) are combined and molecular weight determined by electrospray MS.

(10) The final [Bpa^1, K(BiotinACA)Nle^{12}]α-factor is obtained in 64% yield starting from Fmoc-BpaHWLQLKPGQPNleY with a purity

of >98%. Similar procedures are used to prepare α-factor with Bpa substituted throughout the peptide sequence.

2.4. Cross-linking conditions for Bpa-labeled peptides

Bpa-containing peptides are readily cross-linked into isolated proteins, into proteins in membranes or into proteins in cells. In the case of photoactive α-factor analogs, the peptide is incubated with yeast membranes or whole cells, photo-activation is carried out, and then the cross-linked peptide–protein complex isolated as detailed below. The following protocol was published in Henry et al. (2002) and Son et al. (2004) and was slightly modified for this chapter.

2.5. Cross-linking of [Bpa^{1}, K(BiotinACA)Nle^{12}]α-factor into Ste2p

(1) Yeast cell membranes are prepared by growing 1–6 L of yeast culture overnight, harvesting by centrifugation (500 × *g*, 5 min), and suspending in Buffer A (10 m*M* HEPES and 4 m*M* EDTA, pH 7.0) with proteinase inhibitors [100× stock: pepstatin (1 mg/mL), leupeptin (1 mg/mL), and PMSF (10 mg/mL)]. [Note: In our hands, cross-linking is more efficient with yeast membranes than with intact yeast cells.]

(2) The cells are homogenized using glass beads with a tissue grinder. The homogenates are centrifuged at 500 × *g* for 5 min at 4 °C to remove debris and unbroken cells. Supernatants are centrifuged at 40,000 × *g* for 30 min at 4 °C, the supernatants discarded, and the membrane pellets suspended in Buffer A with the same proteinase inhibitors. The enriched membrane fractions are stable for at least 3 weeks under these conditions.

(3) Yeast cell membranes (220 μg/mL of total protein) are incubated with PPBi buffer (975 μL; 0.1% bovine serum albumin (BSA)) in siliconized microfuge tubes at room temperature (10 min).

(4) [Bpa^{1}, K(BiotinACA)Nle^{12}]α-factor (10 n*M*) is added, and the reaction mixture is gently mixed for 30 min at room temperature.

(5) The reaction mixture is aliquoted (~300 μL/well) into a chilled 24-well plastic culture plate preblocked with PPBi buffer [0.5 *M* potassium phosphate at pH 6.2, 10 m*M* *tert*-amyl methyl ether, 10 m*M* sodium azide, 10 m*M* potassium fluoride, and 0.1% BSA].

(6) The reaction mixture is divided so as to keep the depth of the samples minimal for efficient UV penetration of the sample.

(7) The samples are held at 4 °C and irradiated without the culture plate lid at 365 nm for 45 min at a distance of 12 cm in a Stratlinker (Stratagene, La Jolla, CA). [Note: The time of irradiation can be varied depending on the affinity of the peptide ligand. We have used times up to 90 min. Longer times can lead to various side reactions and heating effects.]

(8) Membrane samples are combined in siliconized microfuge tubes and washed twice by centrifugation ($14{,}000 \times g$) with PPBi (0.1% BSA).

(9) Membrane pellets are dissolved in 5 μL of sample buffer [0.25 *M* Tris–HCl (pH 8.8), 0.005% bromophenol blue, 5% glycerol, 1.25% β-mercaptoethanol, and 2% SDS], heated to 37 °C for 10 min and separated by SDS-PAGE (12% Bis–Tris gels, Invitrogen, 30 mA).

(10) The cross-linked samples resolved with SDS-PAGE are transferred to a PVDF membrane and assayed for the detection of covalently linked [Bpa^{1}, K(BiotinACA)Nle^{12}]α-factor with the NeutrAvidin-HRP conjugate (NA-HRP) (Pierce). All the gels used in these blots are also stained with Coomassie Blue to ensure efficient transfer of the protein to the membrane. Cross-linked receptor is treated further as described below.

3. CHEMICAL CROSS-LINKING

Since the seminal report by Singer and coworkers in 1962 (Wofsy, Metzger, & Singer, 1962), chemical affinity labeling has been used extensively by biochemists. In most cases, an electrophilic center is attacked by a nucleophilic side chain in the protein of interest. A major disadvantage of most affinity reagents is that they are always active and therefore can lead to side reactions. In contrast, 3,4-dihydroxyphenyl groups are only active after conversion to the orthoquinone by oxidation with periodate. Studies by the Kodadek group provided fundamental information on the use of 3,4-dihydroxyphenylalanine (DOPA) and periodate oxidation to cross-link proteins and showed that Cys side chains were among the most active nucleophiles in cross-linking reactions (Burdine, Gillette, Lin, & Kodadek, 2004; Liu, Burdine, & Kodadek, 2006). We have found that the DOPA group is an excellent precursor for cross-linking of α-factor into Ste2p, and in principal this should apply to cross-linking of DOPA-containing peptides into any membrane protein. DOPA is similar in size to tyrosine and can also be used to replace phenylalanine and tryptophan residues. A cartoon

representing the cross-linking and analysis of DOPA peptides into Ste2p is shown in Fig. 1.

3.1. Synthesis of DOPA-biotinylated peptides

DOPA peptides can be synthesized using the procedure used above for Bpa peptides. Fmoc-DOPA (acetonide) is commercially available from Novabiochem (EMD-Millipore, cat. #852093) and may be used with standard synthesizers. If the DOPA residue is at the N-terminus, it is possible to use the less-expensive Fmoc-DOPA without protection (AnaSpec cat #26075). The acetonide derivative is stable to standard peptide synthesis conditions and is readily removed during the TFA cleavage of the peptide from the WANG resin to give Fmoc-DOPA-containing peptide. The following protocol was published in Umanah et al. (2009, 2010) and was slightly modified for this chapter.

3.2. Synthesis of [DOPA^{1},Lys^{7}(BioACA),Nle^{12}]α-factor

Note: DOPA is susceptible to air oxidation. To avoid this, a blanket of argon is used. We routinely run the reaction protected from light.

(1) Fmoc-DOPA-HWLQLKPGQPNleY-OH (26 mg, 12.4 μmol) is dissolved in DMF (2 mL) and sodium borate (1 mL, 50 m*M*).

(2) Biotinamidohexanoic acid *N*-hydroxysuccinimide ester (8.4 mg, 18.6 μmol) is added and the solution is stirred for 2 h at 4 °C.

(3) The reaction is monitored and worked up as described for the analogous Bpa-containing peptide (see Section 2.3).

(4) An 80% yield of a highly homogeneous [DOPA^{1},Lys^{7}(BioACA), Nle^{12}]α-factor is obtained. MW: Calc., 1998.2; found, 1998.2. [MS profiles are usually clean. Small amounts of a product with a mass of −2 Da compared with theoretical may be observed due to presence of the orthoquinone.]

3.3. Cross-linking conditions for DOPA-labeled peptides

Cross-linking of the DOPA-containing peptide uses periodate oxidation following procedures developed by Kodadek and coworkers (Archer, Burdine, & Kodadek, 2005; Burdine et al., 2004; Liu et al., 2006). The following protocol was published in Umanah et al. (2009, 2010) and was slightly modified for this chapter.

(1) Yeast cells expressing C-terminal FLAG and His-tagged Ste2p are grown and total cell membranes are enriched as described above.

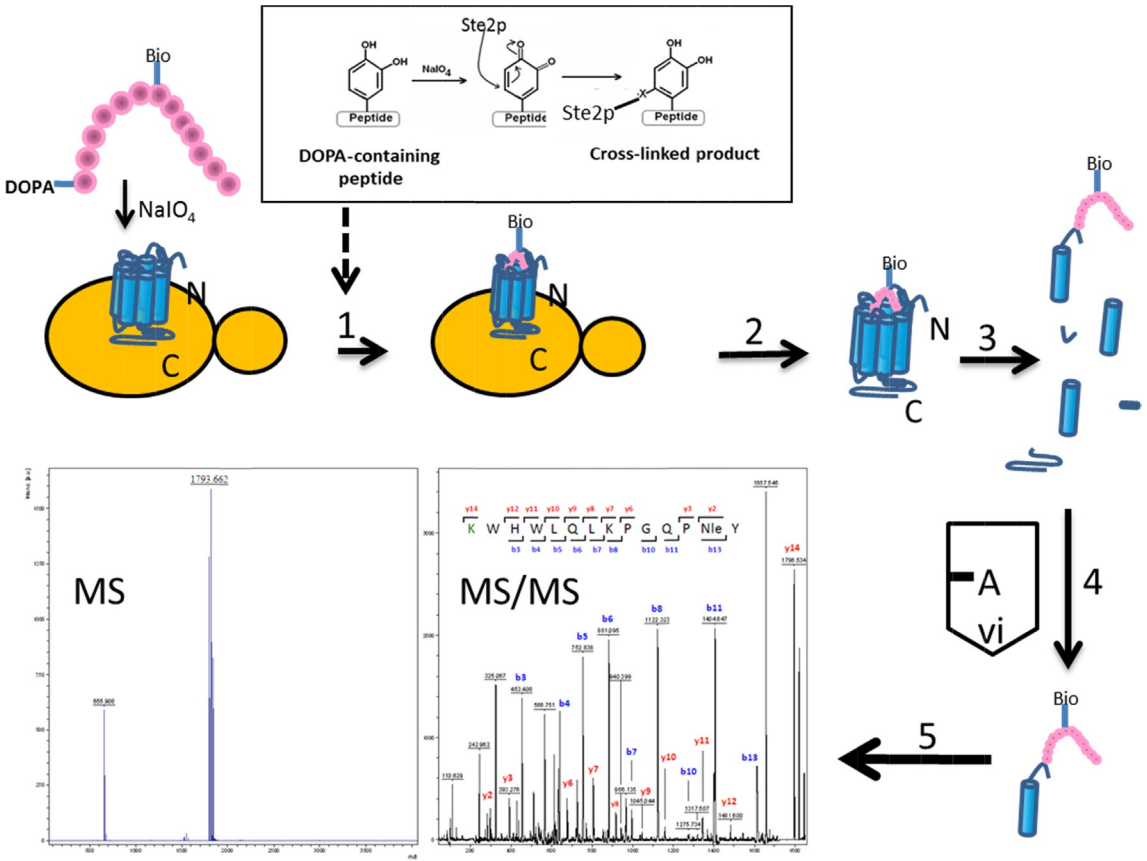


Figure 1 Yeast cells expressing Ste2p are incubated with the DOPA-labeled biotinylated ligand. Cross-linking of the ligand into the receptor (Step 1) occurs after addition of sodium periodate by a reaction represented in the box in the figure. Step 2 represents solubilization of Ste2p and Step 3 indicates digestion of the ligand–receptor complex by CNBr. The ligand-labeled Ste2p fragment is enriched by avidin affinity chromatography (Step 4) and analyzed by MS or MS/MS (Step 5).

(2) Protein concentration is determined using the Bio-Rad (Bio-Rad, Hercules, CA) protein assay reagent.

(3) The membranes are resuspended in NE buffer (20 m*M* HEPES, 20% glycerol, 100 m*M* KCl, 12.5 m*M* EDTA, 0.5 m*M* DTT) and incubated with [DOPA^{1},Lys^{7}(BioACA),Nle^{12}]α-factor (1 μ*M*).

(4) $NaIO_4$ is added to the membrane mixture (final concentration 1.0 m*M*) and oxidation proceeds for 2 min. The reaction is quenched with 1,4-dithiothreitol (100 m*M* final concentration).

(5) Membranes are washed three times with CAPS buffer (*N*-cyclohexyl-3-aminopropanesulfonic acid (Sigma, St. Louis, MO), 10 m*M*, pH 10) by centrifugation to remove un-cross-linked [DOPA^{1},Lys^{7}(BioACA),Nle^{12}]α-factor, the membrane samples are fractionated by SDS-PAGE and then immuno-blotted with an antibody directed against the N-terminal 60 amino acids of Ste2p and with neutravidin-HRP conjugate (Pierce, Rockford, IL) to detect the biotin tag incorporated into Ste2p by covalent linkage to the biotinylated pheromone.

(6) The signals generated are analyzed using Quantity One software (version 4.5.1) on a Chemi-Doc XRS photodocumentation system (Bio-Rad, Hercules, CA).

4. CROSS-LINKING BY UNNATURAL AMINO ACID REPLACEMENT INTO THE RECEPTOR

In the last decade, a number of laboratories have developed means to insert unnatural amino acids into proteins by genetic engineering. These techniques utilize the ability of the living cell, and in some cases of *in vitro* systems, to recognize tRNA and tRNA synthetases that have been mutated to accept unnatural amino acids that are inserted into mutated codons at any position of a gene encoding a protein. Thus, a receptor can be engineered to incorporate a variety of unnatural amino acids (amino acids with side chains that are not found naturally in proteins). These amino acids may contain side chains with a variety of functional groups including fluorescent moieties, chemically reactive groups, and photochemically activatable groups. The position of the unnatural amino acid in the mutated receptor is controlled by the position of the mutated codon in the encoding gene.

Following the procedure developed in the lab of Peter Schultz (Chin et al., 2003), we have used UAAR to insert Bpa into Ste2p at a number

of positions. Below, we outline the procedure used to insert Bpa into Ste2p for the subsequent photochemically activated capture of a Ste2p ligand. The position of Bpa required to capture the ligand helped to identify a ligand-binding site within Ste2p. The following protocol was published in Huang et al. (2008) and was slightly modified for this chapter. The box below outlines the overall steps for this protocol.

4.1. Ligand capture by unnatural amino acid replacement in Ste2p

1. Site-directed mutagenesis of plasmid-borne *STE* target gene replaces selected codon by TAG (Stop codon).
2. Mutated plasmid is transformed into a yeast *ste2-deletion* strain that contains another plasmid encoding an aminoacyl-tRNA synthetase and an orthogonal tRNA that recognizes both a specific unnatural amino acid (Bpa) and the TAG codon.
3. The unnatural amino acid Bpa for the orthogonal tRNA–tRNA synthetase pair is added to the culture.
4. Bpa incorporates into Ste2p at TAG codon.
5. The yeast expressing this mutated protein is assayed for receptor activity.
6. Ste2p is purified, and the incorporation of Bpa into Ste2p is determined by MS.
7. The Bpa-Ste2p is now used to capture the ligand.

4.2. Incorporation of the unnatural amino acid Bpa into Ste2p

(1) A plasmid containing the *STE2* gene and a selectable marker for growth in the recipient yeast strain is engineered to incorporate a TAG stop codon at a specific position within the *STE2* coding region by single-stranded mutagenesis.

(2) The sequence of all TAG mutants is verified by DNA sequence analysis.

(3) The mutant plasmid is co-transformed into yeast cells along with the plasmid pECTyrRS/tRNACUA (Chin et al., 2003) encoding the orthogonal amber suppressor tRNA synthetase/tRNA pair genetically modified to allow for incorporation of Bpa (Chen, Schultz, & Brock, 2007).

(4) Transformants are selected by growth on minimal medium lacking the selectable markers for the plasmids containing the *STE2* and tRNA/tRNA synthetase genes to maintain selection for the plasmids.

(5) For incorporation of Bpa into Ste2p, cells are grown with shaking (200 rpm) in the presence of 2 m*M* Bpa (added from a solution of 100 m*M* in 1 N NaOH) to mid-log phase at room temperature.

5. IDENTIFYING THE CROSS-LINK SITE

Once the peptide has been cross-linked to the receptor by either photochemical or chemical reactions, experiments may be initiated to define the contact point between protein and ligand. These experiments are facilitated by first enriching the receptor–ligand complex using either gel electrophoresis followed by electroelution or affinity techniques, then fragmenting the complex using chemical or enzymatic cleavage reactions with subsequent identification of the cross-linked fragment by SDS-PAGE enrichment and high-resolution mass spectrometry. In the following method, we use affinity enrichment followed by chemical fragmentation followed by mass spectrometry to identify the cross-linked site. The following protocol was published in Umanah et al. (2010) and was slightly modified for this chapter.

5.1. Isolating the tagged receptor–ligand complex

Enrichment of Ste2p cross-linked to [DOPA^{1},Lys^{7}(BioACA),Nle^{12}]α-factor.

(1) Approximately 10 mg of cell membrane containing (BioDOPA)α-factor cross-linked Ste2p is resuspended in ice-cold solubilization buffer (50 m*M* Tris–HCl, pH 7.4, 150 m*M* NaCl, 1% Triton X-100) with protease inhibitors (100× stock: pepstatin (1 mg/mL), leupeptin (1 mg/mL), and PMSF (10 mg/mL)) and incubated overnight at 4 °C with end-over-end mixing and then centrifuged at 15,000 × *g* for 30 min to remove insoluble material.

(2) The solubilized proteins are mixed with His-HC-nickel gel (Sigma or Qiagen) and incubated at 4 °C with end-over-end mixing for 1 h and the gel collected by centrifugation at low speed (500 × *g*, 1 min) and resuspended and washed (4 ×) by centrifugation in buffer (50 m*M* sodium phosphate, pH 8.0, 0.3 *M* sodium chloride, and 5 m*M* imidazole).

(3) The cross-linked receptor peptide complex is eluted by resuspending the resin in 1 mL of ice-cold elution buffer (50 m*M* sodium phosphate, pH 8.0, 0.3 *M* sodium chloride, and 250 m*M* imidazole) and incubated at 4 °C with end-over-end mixing for 10 min.

(4) The resin is pelleted by centrifugation (2000 × *g*, 1 min) and the supernatant, containing the eluted Ste2p, transferred to a fresh tube.
(5) Purity and concentration of samples are estimated by Coomassie blue and silver staining of SDS-PAGE gels.
(6) The enrichment of cross-linked Ste2p is confirmed by immunoblotting using an antibody directed against Ste2p and with neutravidin-HRP conjugate to detect the biotin tag covalently inserted into the receptor.

5.2. Fragmenting cross-linked peptide–protein complex

We have employed trypsin, cyanogen bromide (CNBr), and BNPS-skatole cleavage to generate receptor fragments. These methods cleave the receptor at Lys/Arg, Met, or Trp residues, respectively. Knowing the cleavage points allows one to generate maps of predicted receptor fragments. Adding the molecular weight of the cross-linked ligand to these fragments allows approximation of the size of the cross-linked fragmentation product to begin to localize the cross-link sites. The following protocol was published in Son et al. (2004) and was slightly modified for this chapter.

(1) Cross-linked samples are digested with CNBr, trypsin, or BNPS-skatole. Conditions for digestion by each reagent are described separately below.
(2) For CNBr digestion, the enriched cross-linked Ste2p samples (~20 μg) eluted from the His-nickel column are dried by vacuum centrifugation (Thermo Scientific, Waltham, MA) and then dissolved in 100% TFA containing 10 mg/mL CNBr. Cleavage may also be performed in 70% formic acid in place of TFA. Deionized distilled water (ddH_2O) is added to adjust the final TFA concentration to 80%.
(3) After incubation of the samples at 37 °C in the dark for 18 h, they are dried by vacuum centrifugation and washed three times with ddH_2O and then 1 *M* Tris (pH 8.0) added to neutralize the acidic mixture.
(4) For trypsin digestion, samples are dissolved in trypsin digestion buffer [100 m*M* Tris–HCl (pH 8.5)], and 6.25 μg of trypsin (sequencing grade modified trypsin, Roche) was added to 250 μg of total protein; after incubation for 6 h, a second batch of trypsin (6.25 μg) is added to achieve complete digestion. Ste2p contains a lysine residue (K269) followed by a proline residue (P270) in extracellular loop 3, which makes it less likely to be digested by trypsin. Nevertheless, we found that this region is reproducibly susceptible to trypsin digestion under the conditions described above.

(5) For BNPS-skatole digestion, samples are dissolved in 50% acetic acid with addition of 10 mg/mL BNPS-skatole. To prevent anomalous cleavage of Ste2p at histidine and tyrosine residues, 100-fold molar excess of tyrosine is added to the reaction mixture during BNPS-skatole digestion.

5.3. Isolating cross-linked fragment

The isolation of the cross-linked ligand–receptor fragments is facilitated by the biotin moiety that is incorporated into the receptor. The extremely high binding affinity of avidin for biotin leads to capture of the fragment on avidin beads even when they are present in minute amounts. In principle, the cross-linked fragments can also be cut from the acrylamide gel and characterized (see below). The following protocol was published in Son et al. (2005) and was slightly modified for this chapter.

(1) Fragments from the CNBr digestion of cross-linked Ste2p are resuspended in PBS buffer (0.1 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7), mixed with monomeric avidin resin (Pierce Thermo Scientific, Rockford, IL), and incubated with end-over-end mixing for 6 h at 4 °C.

(2) The resin is collected by centrifugation at low speed (1000 × *g*, 1 min) and washed 4 × by resuspending in PBS buffer.

(3) The cross-linked Ste2p fragments is eluted from the resin by resuspending the resin in 200 μL of ice-cold elution buffer (0.1 *M* glycine, pH 2.5) and incubating at 4 °C with end-over-end mixing for 5 min.

(4) The resin is pelleted by centrifugation (2000 × *g*, 1 min) and the supernatant, containing the eluted cross-linked Ste2p fragments, transferred to a fresh tube containing 20 μL of TBS (0.5 *M* Tris–HCl, pH 7.4, 1.5 *M* NaCl).

(5) These samples are used for MS analyses (as below).

5.4. Mass spectrometric analysis of the cross-linked fragment

5.4.1 Matrix-assisted laser desorption ionization-time of flight

To determine whether Bpa is incorporated into Ste2p and to determine the fragment of Ste2p cross-linked to the ligand, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) may be used. For determination of the specific residues of the ligand and the receptor that are cross-linked, tandem mass spectrometry (MS/MS) is preferred (see below). The following

protocol was published in Son et al. (2005) and was slightly modified for this chapter.

(1) The samples are 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).

(2) *R*-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) is used as the matrix. The digested samples (0.5 μL eluate from ZipTip) are either mixed with 0.5 μL of matrix before spotting on the target or 1.0 μL of matrix is spotted and allowed to dry before applying 1.0 μL of samples.

(3) MADLI-TOF spectra are acquired on a Bruker Daltonics (Boston, MA) Microflex using both reflector and linear methods.

(4) The MALDI-TOF spectrum can be used to determine if Bpa is incorporated into Ste2p. The mass found in the MALDI-TOF spectrum of a Ste2p fragment with Bpa incorporated in place of the naturally occurring amino acid should be equivalent to the fragment with the mass of Bpa in place of the mass of the natural amino acid. The mass equivalent to the natural sequence of the Ste2p fragment should be missing in the MALDI-TOF spectrum.

(5) The MALDI-TOF spectrum can also be used to determine which fragment of Ste2p has been cross-linked to the synthetic ligand. A cross-linked fragment should have the combined mass of the Ste2p fragment and the ligand, although a slight loss of mass may occur dependent of the chemistry of the cross-linking reaction.

5.4.2 Tandem mass spectrometry (to determine the cross-link site)

The following protocol was published in Umanah et al. (2010) and was slightly modified for this chapter.

(1) Ste2p digestion fragments are analyzed by reverse-phase microcapillary LC-ESI-MS/MS using a fritless, microcapillary column (100 μm inner diameter) packed with 10 cm of 5-μm C_{18} reverse-phase material (Synergi 4μ Hydro RP80a, Phenomenex).

(2) The digested peptides are loaded onto the reverse-phase column equilibrated in buffer (0.1% formic acid and 5% acetonitrile).

(3) The column is placed in line with a nanoESI-LTQ-Orbitrap mass spectrometer (Thermo Scientific, Inc.). Peptides are eluted using a 100-min linear gradient from 0% to 55% solution (0.1% formic acid, 80% acetonitrile) at a flow rate of 0.3 μL/min.

(4) Eluting peptides are electrosprayed into the mass spectrometer with an applied spray voltage of 2.2 kV.

(5) During the gradient, the eluting ions are analyzed by one full precursor MS scan (400–2000 m/z), followed by five MS/MS scans on the five most abundant ions detected in the precursor MS scan while operating under dynamic exclusion. During the LC/MS/MS analysis of the sample, the precursor ions are analyzed in the Orbitrap, and MS/MS data are analyzed in the ion trap analyzer. The ion trap is more sensitive, enabling the detection of the MS/MS fragment ions, but it has somewhat lower precision than the Orbitrap.

(6) All the acquired MS/MS data are searched against the *Saccharomyces cerevisiae* protein data base using the SEQUEST algorithm (Lundgren, Martinez, Wright, & Han, 2009).

(7) To identify the MS/MS spectra of the cross-linked fragment, the free ligand spectra are compared with the spectra generated from the cross-linked samples eluted from the avidin column.

(8) The Ste2p fragment ions are identified on the cross-linked spectra by comparing the masses of fragment ions that do not correspond to any of ligand fragment ions with predicted masses of Ste2p fragment ions using PROWL MS/MS peptide and protein fragmentation tools (Beavis & Fenyo, 2004).

(9) A series of MS fragments incorporating residues from the ligand and the protein is evidence for a contact point between the peptide and the receptor.

(10) This is verified by repeating the cross-linking with mutations at the putative cross-link site.

6. CONCLUDING REMARKS

Photoaffinity probes have been used extensively to map ligand-binding sites in GPCRs (Grunbeck & Sakmar, 2013), and mass spectrometry has been utilized to determine the specific residue on the GPCR that is cross-linked to the photoaffinity ligand (Umanah et al., 2010). In this review, we have outlined the specific steps that we have used to elucidate residue-to-residue contacts between a peptide ligand and a GPCR using the yeast *S. cerevisiae* as a model system. The methods we used should be applicable to studying other GPCRs, since many GPCRs including human GPCRs may be expressed in yeast (Evans et al., 2009; Minic, Sautel, Salesse, & Pajot-Augy, 2005; Pausch, 1997). They are also applicable to

GPCRs expressed in bacteria, insect cells, and other organisms including mammalian tissue cultures. A significant challenge of this approach is that cross-linked peptides are difficult to isolate because they are often produced in very small quantities and often contain difficult to handle hydrophobic regions of the receptor. From this perspective, we find higher levels of cross-linking with oxidative cross-linking of DOPA than with UV-activated Bpa. Care must be taken with mass spectrometric analysis because cross-linked ligand-GPCR fragments are complex and difficult to analyze unless high-resolution mass spectrometry is used.

A "reverse" system for determination of residues in ligands and receptors that are in contact takes advantage of replacement of natural amino acids in GPCRs with the unnatural amino acid Bpa. This method, developed in the lab of Peter Schultz for yeast (Chen et al., 2007; Liu & Schultz, 2010; Wang, Xie, & Schultz, 2006) can be used for other GPCRs expressed in yeast and can be extended to mammalian GPCRs expressed in bacteria and tissue culture (Niu, Schultz, & Guo, 2013; Ryu & Schultz, 2006) Challenges that exist for UAAR include the lack of expression of some STOP codon engineered in GPCRs and a low incorporation rate of the unnatural amino acid into some STOP codons. These challenges are being met by improved UAAR constructs being developed in several laboratories (Chin, 2014). Despite the above challenges, the methods presented in this chapter have revealed remarkable insights into ligand–GPCR interactions. We look forward to seeing them further contribute to the understanding of the structure and function of GPCRs.

ACKNOWLEDGMENTS

This work was supported by grant GM-22087 from the National Institute of General Medical Sciences of the National Institutes of Health. We gratefully appreciate our hard-working students, fellows, and staff members who have contributed to research in our labs. This review was made possible by the contributions of our dedicated colleagues. We thank Boris Ashava for providing specific details on the synthesis of the probes.

REFERENCES

Akal-Strader, A., Khare, S., Xu, D., Naider, F., & Becker, J. M. (2002). Residues in the first extracellular loop of a G protein-coupled receptor play a role in signal transduction. *The Journal of Biological Chemistry*, *277*(34), 30581–30590.

Archer, C. T., Burdine, L., & Kodadek, T. (2005). Identification of Gal4 activation domain-binding proteins in the 26S proteasome by periodate-triggered cross-linking. *Molecular BioSystems*, *1*(5–6), 366–372.

Beavis, R., & Fenyo, D. (2004). Finding protein sequences using PROWL. *Current Protocols in Bioinformatics*. http://dx.doi.org/10.1002/0471250953.bi1302s7, Chapter 13, Unit 13.12.

Bortolato, A., Dore, A. S., Hollenstein, K., Tehan, B. G., Mason, J. S., & Marshall, F. H. (2014). Structure of Class B GPCRs: New horizons for drug discovery. *British Journal of Pharmacology*, *171*, 3132–3145.

Burdine, L., Gillette, T. G., Lin, H. J., & Kodadek, T. (2004). Periodate-triggered cross-linking of DOPA-containing peptide-protein complexes. *Journal of the American Chemical Society*, *126*(37), 11442–11443.

Burkholder, A. C., & Hartwell, L. H. (1985). The yeast alpha-factor receptor: Structural properties deduced from the sequence of the STE2 gene. *Nucleic Acids Research*, *13*(23), 8463–8475.

Chen, S., Schultz, P. G., & Brock, A. (2007). An improved system for the generation and analysis of mutant proteins containing unnatural amino acids in Saccharomyces cerevisiae. *Journal of Molecular Biology*, *371*(1), 112–122.

Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., et al. (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science*, *318*(5854), 1258–1265.

Chien, E. Y., Liu, W., Zhao, Q., Katritch, V., Han, G. W., Hanson, M. A., et al. (2010). Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science*, *330*(6007), 1091–1095.

Chin, J. W. (2014). Expanding and reprogramming the genetic code of cells and animals. *Annual Review of Biochemistry*, *83*, 379–408.

Chin, J. W., Cropp, T. A., Anderson, J. C., Mukherji, M., Zhang, Z., & Schultz, P. G. (2003). An expanded eukaryotic genetic code. *Science*, *301*(5635), 964–967.

Chung, K. Y., Rasmussen, S. G., Liu, T., Li, S., DeVree, B. T., Chae, P. S., et al. (2011). Conformational changes in the G protein Gs induced by the beta2 adrenergic receptor. *Nature*, *477*(7366), 611–615.

Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991). Model systems for the study of seven-transmembrane-segment receptors. *Annual Review of Biochemistry*, *60*, 653–688.

Dorman, G., & Prestwich, G. D. (1994). Benzophenone photophores in biochemistry. *Biochemistry*, *33*(19), 5661–5673.

Evans, B. J., Wang, Z., Broach, J. R., Oishi, S., Fujii, N., & Peiper, S. C. (2009). Expression of CXCR4, a G-protein-coupled receptor for CXCL12 in yeast identification of new-generation inverse agonists. *Methods in Enzymology*, *460*, 399–412.

Fillion, D., Cabana, J., Guillemette, G., Leduc, R., Lavigne, P., & Escher, E. (2013). Structure of the human angiotensin II type 1 (AT1) receptor bound to angiotensin II from multiple chemoselective photoprobe contacts reveals a unique peptide binding mode. *The Journal of Biological Chemistry*, *288*(12), 8187–8197.

Fredriksson, R., Lagerstrom, M. C., Lundin, L. G., & Schioth, H. B. (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Molecular Pharmacology*, *63*(6), 1256–1272.

Garland, S. L. (2013). Are GPCRs still a source of new targets? *Journal of Biomolecular Screening*, *18*(9), 947–966.

Grunbeck, A., Huber, T., Sachdev, P., & Sakmar, T. P. (2011). Mapping the ligand-binding site on a G protein-coupled receptor (GPCR) using genetically encoded photo-crosslinkers. *Biochemistry*, *50*(17), 3411–3413.

Grunbeck, A., Huber, T., & Sakmar, T. P. (2013). Mapping a ligand binding site using genetically encoded photoactivatable crosslinkers. *Methods in Enzymology*, *520*, 307–322.

Grunbeck, A., & Sakmar, T. P. (2013). Probing G protein-coupled receptor-ligand interactions with targeted photoactivatable cross-linkers. *Biochemistry*, *52*(48), 8625–8632.

Hanson, M. A., & Stevens, R. C. (2009). Discovery of new GPCR biology: One receptor structure at a time. *Structure*, *17*(1), 8–14.

Hauser, M., Kauffman, S., Lee, B. K., Naider, F., & Becker, J. M. (2007). The first extracellular loop of the Saccharomyces cerevisiae G protein-coupled receptor Ste2p undergoes a conformational change upon ligand binding. *The Journal of Biological Chemistry*, *282*(14), 10387–10397.

Henry, L. K., Khare, S., Son, C., Babu, V. V., Naider, F., & Becker, J. M. (2002). Identification of a contact region between the tridecapeptide alpha-factor mating pheromone of Saccharomyces cerevisiae and its G protein-coupled receptor by photoaffinity labeling. *Biochemistry*, *41*(19), 6128–6139.

Huang, L. Y., Umanah, G., Hauser, M., Son, C., Arshava, B., Naider, F., et al. (2008). Unnatural amino acid replacement in a yeast G protein-coupled receptor in its native environment. *Biochemistry*, *47*(20), 5638–5648.

Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y., Lane, J. R., et al. (2008). The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science*, *322*(5905), 1211–1217.

Katritch, V., Cherezov, V., & Stevens, R. C. (2012). Diversity and modularity of G protein-coupled receptor structures. *Trends in Pharmacological Sciences*, *33*(1), 17–27.

Kim, K. M., Lee, Y. H., Akal-Strader, A., Uddin, M. S., Hauser, M., Naider, F., et al. (2012). Multiple regulatory roles of the carboxy terminus of Ste2p a yeast GPCR. *Pharmacological Research*, *65*(1), 31–40, 2.

Kim, H., Lee, B. K., Naider, F., & Becker, J. M. (2009). Identification of specific transmembrane residues and ligand-induced interface changes involved in homo-dimer formation of a yeast G protein-coupled receptor. *Biochemistry*, *48*(46), 10976–10987.

Lappano, R., & Maggiolini, M. (2011). G protein-coupled receptors: Novel targets for drug discovery in cancer. *Nature Reviews Drug Discovery*, *10*(1), 47–60.

Liu, B., Burdine, L., & Kodadek, T. (2006). Chemistry of periodate-mediated cross-linking of 3,4-dihydroxylphenylalanine-containing molecules to proteins. *Journal of the American Chemical Society*, *128*(47), 15228–15235.

Liu, C. C., & Schultz, P. G. (2010). Adding new chemistries to the genetic code. *Annual Review of Biochemistry*, *79*, 413–444.

Lundgren, D. H., Martinez, H., Wright, M. E., & Han, D. K. (2009). Protein identification using Sorcerer 2 and SEQUEST. *Current Protocols in Bioinformatics*, Chapter 13, Unit 13.3.

Minic, J., Sautel, M., Salesse, R., & Pajot-Augy, E. (2005). Yeast system as a screening tool for pharmacological assessment of g protein coupled receptors. *Current Medicinal Chemistry*, *12*(8), 961–969.

Nakayama, N., Miyajima, A., & Arai, K. (1985). Nucleotide sequences of STE2 and STE3, cell type-specific sterile genes from Saccharomyces cerevisiae. *The EMBO Journal*, *4*(10), 2643–2648.

Niu, W., Schultz, P. G., & Guo, J. (2013). An expanded genetic code in mammalian cells with a functional quadruplet codon. *ACS Chemical Biology*, *8*, 1640–1645.

Pausch, M. H. (1997). G-protein-coupled receptors in Saccharomyces cerevisiae: High-throughput screening assays for drug discovery. *Trends in Biotechnology*, *15*(12), 487–494.

Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., et al. (2011). Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature*, *477*(7366), 549–555.

Raths, S. K., Naider, F., & Becker, J. M. (1988). Peptide analogues compete with the binding of alpha-factor to its receptor in Saccharomyces cerevisiae. *The Journal of Biological Chemistry*, *263*(33), 17333–17341.

Rihakova, L., Deraet, M., Auger-Messier, M., Perodin, J., Boucard, A. A., Guillemette, G., et al. (2002). Methionine proximity assay, a novel method for exploring peptide ligand-receptor interaction. *Journal of Receptor and Signal Transduction Research*, *22*(1–4), 297–313.

Ryu, Y., & Schultz, P. G. (2006). Efficient incorporation of unnatural amino acids into proteins in Escherichia coli. *Nature Methods*, *3*(4), 263–265.

Scheerer, P., Heck, M., Goede, A., Park, J. H., Choe, H. W., Ernst, O. P., et al. (2009). Structural and kinetic modeling of an activating helix switch in the rhodopsin-transducin interface. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(26), 10660–10665.

Son, C. D., Sargsyan, H., Hurst, G. B., Naider, F., & Becker, J. M. (2005). Analysis of ligand-receptor cross-linked fragments by mass spectrometry. *The Journal of Peptide Research*, *65*(3), 418–426.

Son, C. D., Sargsyan, H., Naider, F., & Becker, J. M. (2004). Identification of ligand binding regions of the Saccharomyces cerevisiae alpha-factor pheromone receptor by photoaffinity cross-linking. *Biochemistry*, *43*(41), 13193–13203.

Sumranjit, J., & Chung, S. J. (2013). Recent advances in target characterization and identification by photoaffinity probes. *Molecules*, *18*(9), 10425–10451.

Umanah, G. K., Huang, L., Ding, F. X., Arshava, B., Farley, A. R., Link, A. J., et al. (2010). Identification of residue-to-residue contact between a peptide ligand and its G protein-coupled receptor using periodate-mediated dihydroxyphenylalanine cross-linking and mass spectrometry. *The Journal of Biological Chemistry*, *285*(50), 39425–39436.

Umanah, G. K., Huang, L. Y., Maccarone, J. M., Naider, F., & Becker, J. M. (2011). Changes in conformation at the cytoplasmic ends of the fifth and sixth transmembrane helices of a yeast G protein-coupled receptor in response to ligand binding. *Biochemistry*, *50*(32), 6841–6854.

Umanah, G. K., Son, C., Ding, F., Naider, F., & Becker, J. M. (2009). Cross-linking of a DOPA-containing peptide ligand into its G protein-coupled receptor. *Biochemistry*, *48*(9), 2033–2044.

Wang, Y., & Dohlman, H. G. (2004). Pheromone signaling mechanisms in yeast: A prototypical sex machine. *Science*, *306*(5701), 1508–1509.

Wang, L., Xie, J., & Schultz, P. G. (2006). Expanding the genetic code. *Annual Review of Biophysics and Biomolecular Structure*, *35*, 225–249.

Warne, T., Moukhametzianov, R., Baker, J. G., Nehme, R., Edwards, P. C., Leslie, A. G., et al. (2011). The structural basis for agonist and partial agonist action on a beta(1)-adrenergic receptor. *Nature*, *469*(7329), 241–244.

Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., et al. (2008). Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature*, *454*(7203), 486–491.

Wittelsberger, A., Mierke, D. F., & Rosenblatt, M. (2008). Mapping ligand-receptor interfaces: Approaching the resolution limit of benzophenone-based photoaffinity scanning. *Chemical Biology & Drug Design*, *71*(4), 380–383.

Wofsy, L., Metzger, H., & Singer, S. J. (1962). Affinity labeling-a general method for labeling the active sites of antibody and enzyme molecules. *Biochemistry*, *1*, 1031–1039.

Wu, B., Chien, E. Y., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., et al. (2010). Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science*, *330*(6007), 1066–1071.

Wu, Y. L., Hooks, S. B., Harden, T. K., & Dohlman, H. G. (2004). Dominant-negative inhibition of pheromone receptor signaling by a single point mutation in the G protein alpha subunit. *The Journal of Biological Chemistry*, *279*(34), 35287–35297.

Wu, H., Wang, C., Gregory, K. J., Han, G. W., Cho, H. P., Xia, Y., et al. (2014). Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator. *Science*, *344*(6179), 58–64.

Xue, C. B., Eriotou-Bargiota, E., Miller, D., Becker, J. M., & Naider, F. (1989). A covalently constrained congener of the Saccharomyces cerevisiae tridecapeptide mating pheromone is an agonist. *The Journal of Biological Chemistry*, *264*(32), 19161–19168.