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Assessment of constitutive activity of a G protein-coupled receptor, Cpr2, in *Cryptococcus neoformans* by heterologous and homologous methods

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Abstract

G protein-coupled receptors (GPCRs) comprise the largest superfamily of cell surface receptors, and are primary targets for drug development. A variety of detection systems have been reported to study ligand-GPCR interactions. Using *Saccharomyces cerevisiae* to express foreign proteins has long been appreciated for its low cost, simplicity, and conserved cellular pathways. The yeast pheromone responsive pathway has been utilized to assess a range of different GPCRs. We have identified a pheromone-like receptor, Cpr2, that is located outside of the *MAT* locus in the human fungal pathogen *Cryptococcus neoformans*. To characterize its function and potential ligands, we expressed *CPR2* in a yeast heterologous expression system. To optimize for *CPR2* expression in this system, pheromone receptor Ste3, regulator of G protein signaling (RGS) Sst2, and the cyclin-dependent kinase inhibitor Far1 were mutated. The *lacZ* gene was fused with the promoter of the *FUS1* gene that is activated by the yeast pheromone signal and then introduced into yeast cells. Expression of *CPR2* in this yeast heterologous expression system revealed that Cpr2 could activate the pheromone responsive pathway without additional of potential ligands, suggesting it is a naturally-occurring, constitutively active receptor. Mutation of a single amino acid, Leu²²², was sufficient to reverse the constitutive activity of Cpr2. In this report, we summarize methods used for assessing the constitutive activity of Cpr2 and its mutants, which could be beneficial for other GPCR studies.

1. Introduction of receptors and constitutive receptors

All living organisms must sense environmental signals and respond appropriately to survive. Sensing the environment depends on cell surface receptors, which include the family of G protein-coupled receptors (GPCRs) (Bahn et al., 2007). GPCRs comprise the largest superfamily of cell surface receptors, which respond to a variety of extracellular stimuli as diverse as hormones, neurochemicals, odorants, nutrients, and light (Bockaert and Pin, 1999). Despite exhibiting diversity in primary sequence and biological function, all GPCRs possess the same fundamental architecture, consisting of seven transmembrane domains (7TMs), and share common mechanisms of signal transduction. These receptors are also of great pharmacological importance (Drews, 1996). To date, almost half of all clinically relevant drugs act as agonist or antagonists of GPCRs (Drews, 1996).

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Generally, GPCRs function as guanine nucleotide exchange factors (GEFs) to activate heterotrimeric G proteins that play a central role in transducing extracellular signals into intrinsic signals and effecting appropriate biochemical and physiological responses. Ligand activation of GPCRs triggers the coupled $G\alpha$ subunit to release GDP and bind GTP, transforming them into the $G\alpha$ -GTP active state. The GTP binding results in a conformational change of the $G\alpha$ subunit, which promotes its dissociation from the $G\beta\gamma$ complex. The liberated $G\alpha$ or $G\beta\gamma$ subunits then interact with downstream effectors to switch on or off signaling cascades. RGS (Regulators of G protein signaling) proteins, on the other hand, negatively regulate the G protein signaling by converting $G\alpha$ protein from GTP-bound state to GDP-bound state.

Much of GPCR research has focused on the effects of agonist stimulation of these GPCRs, but it has become clear that many GPCRs exist in a constitutively active state, where activation occurs in the absence of an agonist. These GPCRs are locked in an active state and active downstream signal in a ligand-independent fashion, and continuously stimulate their intracellular signaling pathways (Rosenkilde et al., 2006). The hypothesis that some GPCRs can signal in the absence of any external ligand was first supported by studies of the opioid and β_2 -adrenergic receptors (Cerione et al., 1984; Koski et al., 1982). Constitutively active GPCRs have been generated artificially by mutagenesis, and also have been reported to exist as naturally occurring point mutations that increase constitutive activity (Van Sande et al., 1995). Moreover, approximately 60 wild-type GPCRs from human, mouse, or rat have been shown to exhibit considerable constitutive activity and many of them found to be linked to human diseases (Seifert and Wenzel-Seifert, 2002). Therefore, constitutively active GPCRs may be of great physiological importance in both natural physiology and disease states.

A pheromone receptor-like gene, *CPR2*, was identified in *Cryptococcus neoformans*, a human fungal pathogen that commonly causes meningoencephalitis mostly in immunocompromised populations (Hsueh et al., 2009). *Cpr2* appears to be a naturally occurring, constitutively active GPCR based on biochemical and genetic evidence: First, expression of *CPR2* in a yeast heterologous expression system revealed that *Cpr2* activates downstream signals without addition of potential ligands; second, a single amino acid, Leu²²², was found to produce its constitutive activity. The constitutively active status of *Cpr2* was further confirmed in phenotypic analysis and protein-protein interaction assays using a split-ubiquitin system (Hsueh et al., 2009). In this chapter, we will discuss several methods used to assess GPCR constitutive activity in fungi using *Cpr2* as an example.

2. Identification of *Cpr2* as a natural occurring constitutively active receptor

2.1 GPCR and G protein signaling in *C. neoformans*

GPCRs have been identified in a variety of fungi and play important roles in cell development and fungal pathogenesis. The model ascomycete *Saccharomyces cerevisiae* has only three GPCRs: two pheromone receptors (*Ste2* and *Ste3*) and one sugar sensor (*Gpr1*) (Burkholder and Hartwell, 1985; Hagen et al., 1986; Kraakman et al., 1999). The pheromones and pheromone receptors are expressed in a cell-type specific manner in *S. cerevisiae*. α cells express the lipopeptide pheromone α -factor and the α -factor receptor *Ste2* whereas α cells express the peptide pheromone α -factor and the α -factor receptor *Ste3*. *Ste2* and *Ste3* activate *Gpa1* G protein to regulate the pheromone-responsive pathway, while *Gpr1* is coupled to *Gpa2* to control the nutrient sensing of yeast cells (Harashima and Heitman, 2004; Lengeler et al., 2000). The *Gpa1*-pheromone response pathway is among the best understood signaling pathways in eukaryotes and serve as a model for GPCR-mediated signaling. Studies of this model have contributed enormously to the understanding of the mechanisms of G protein signaling and regulation. The yeast pheromone pathway has also

been developed as a heterologous expression system to identify the potential agonists and antagonists for a receptor (Mentesana et al., 2002; Xue et al., 2008b).

In the basidiomycete *C. neoformans*, three G protein α subunits control two major G protein-signaling pathways that are important for cell development and fungal virulence. One G α protein, Gpa1, activates the nutrient sensing pathway by regulating the activity of protein kinase A (PKA) and controls fungal virulence (Alspaugh et al., 1997; Xue et al., 2008a). The receptors for activating this nutrient signaling pathway remain to be understood even though one GPCR, Gpr4, has been found to be involved in this activation, probably by sensing amino acids (Xue et al., 2006). Two other G α subunits, Gpa2 and Gpa3, are both involved in the pheromone sensing pathway in *C. neoformans*. *C. neoformans* has a defined sexual cycle and a simple bipolar mating system (**a** and α) in which a single mating-type (*MAT*) locus spans an approximately 120 kb recombinationally-suppressed region (Hull et al., 2002). Only **a**-factor-like lipopeptide pheromones and Ste3-like pheromone receptors are known in *C. neoformans*, neither an α -like pheromone gene nor a Ste2-like ortholog is apparent in any of sequenced genomes. Pheromone receptors Ste3 α and Ste3**a** are encoded in the *MAT* locus and sense pheromones from cells of the opposite mating type and activate a G-protein complex that includes Gpa2 and Gpa3, the G β subunit Gbp1, and the G γ subunits Gpg1 and Gpg2 (Hsueh et al., 2007; Li et al., 2007). Following activation of Gpa2 and Gpa3, the G β subunits Gbp1 is released to activate the downstream MAPK cascade to trigger mating responses.

2.2. Identification of Cpr2 protein

A Ste3-like protein (CNAG_03938) was identified in our search for potential GPCR proteins in *C. neoformans* H99 genome, and was named as Cpr2 (*Cryptococcus* Pheromone Receptor 2) (Hsueh et al., 2009). Cpr2 contains 414 amino acids and 7 predicted transmembrane domains (TMs), a structural hallmark of GPCRs. The *MAT* locus of *C. neoformans* has been extensively studied and among 20 or so genes encoded by this locus, the homeodomain proteins and the pheromone/pheromone receptor genes establish the sexual identity of a cell (Chung et al., 2002; Hull et al., 2005). Interestingly, Cpr2 is not located in the *MAT* locus. A search for Ste3-like receptors in other basidiomyceteous fungi revealed that many species also have additional pheromone receptor paralogs that are not part of *MAT* (Aimi et al., 2005; James et al., 2006), suggesting basidiomyceteous fungi may have an additional mechanism for pheromone sensing and regulation.

Functional studies revealed that Cpr2 expression is regulated by pheromone and highly induced during mating. A strain carrying a deletion mutation of *CPR2* is still fertile but has a defect in cell fusion. However, overexpression of *CPR2* elicits unisexual mating (Hsueh et al., 2009). To understand whether this protein functions as a pheromone sensor, we expressed *CPR2* gene in a yeast heterologous expression system.

2.3. Expression of *CPR2* in a yeast heterologous expression system

In order to better understand the function of GPCRs, researchers attempt to express the proteins in less sophisticated organisms. Even though GPCRs in fungi have not yet been developed as drug targets for disease control, GPCR-related signaling pathways have been utilized to de-orphanize GPCRs (Mentesana et al., 2002). A bioassay based on the *S. cerevisiae* pheromone response pathway has been developed to characterize heterologous GPCRs. However, several preconditions must be met to utilize this pathway. First, proper plasma membrane expression of the target GPCR is needed; second, the foreign receptor must properly couple to the G α protein Gpa1 to activate the downstream signal; third, a reporter gene in this pathway is needed to monitor GPCR expression and signal activation. Finally, the pathway needs to be optimized to enhance signal sensitivity.

Some foreign GPCR genes can be directly expressed in *S. cerevisiae* and coupled to the Gpa1 G protein to activate the yeast pheromone responsive pathway, including some pheromone receptors from *S. commune* (Fowler et al., 1999), the mammalian SSTR₂ receptor (Price et al., 1995), the adenosine A₂ receptor (Price et al., 1996), the melatonin Mel_{1a} receptor (Kokkola et al., 1998), and the UDP-glucose receptor (Chambers et al., 2000). We also expressed the *C. neoformans* *CPR2* gene in this *Saccharomyces* heterologous system to identify its potential ligands (Hsueh et al., 2009).

2.3.1 Establishment of the yeast heterologous expression system—To engineer the *S. cerevisiae* pheromone response pathway as a heterologous expression system, several modifications are necessary to optimize signal output. The cyclin-dependent kinase inhibitor Far1 promotes yeast cell arrest in G1 in response to pheromone (Chang and Herskowitz, 1990), and *FAR1* is deleted to allow continued cell division in cells responding to pheromone or a heterologous ligand. The regulator of G protein signaling (RGS) Sst2 functions as a negative regulator of Gpa1 activity and deletion of *SST2* significantly increases the sensitivity of pheromone response (Dietzel and Kurjan, 1987) and the yeast heterologous GPCR expression signal. To monitor the pheromone response signal for large scale screens, the pheromone inducible gene *FUS1* (Hagen et al., 1991) is commonly fused with either the *lacZ* gene or the *HIS3* gene. Finally, to avoid interference by the endogenous receptors, the yeast pheromone receptor gene *STE2* or *STE3* is deleted (Figure 1).

Required materials: Vectors and strains used are listed in Table 1.

Vectors: pTCFL1 is a *S. cerevisiae* expression vector that contains a *FUS1-lacZ* fusion (Chen and Kurjan, 1997). When the pheromone responsive pathway is activated, the *FUS1-lacZ* fusion will express and beta-galactosidase activity can be measured. pPGK is a *S. cerevisiae* overexpression vector that contains the yeast phosphoglycerate kinase (PGK) promoter (Kang et al., 1990).

***S. cerevisiae* strains:** W303-1A (*MATa*) and W303-1B (*MATa*) are laboratory strains used in this assay. SDK45 was generated based on W303-1B by deleting pheromone receptor *STE3*, and SDK47 was generated based on SDK45 by further deleting RGS gene *SST2* (Fowler et al., 1999). The *FAR1* gene was mutated in SDK47 to generate YDX4.

Media: YPD rich medium, SD-Trp, and SD-Trp-Ura drop out media were prepared as described (Amberg et al., 2005). To prepare 200 ml of 5x Z-buffer, 16.1 g Na₂HPO₄·7H₂O, 6.23 g NaH₂PO₄·2H₂O, 0.75 g KCl, 0.246 g MgSO₄·7H₂O are added and adjusted to pH 7.0, then add H₂O to a total volume of 200 mL. Z-buffer should be stored at 4°C.

Chemicals: Lithium acetate (Fisher Scientific), Salmon sperm single-stranded DNA (Sigma), CPRG (chlorophenolred-β-D-galactopyranoside, CalBiochem), β-mercaptoethanol (Sigma). Synthetic mating pheromones MFa and MFα (Heitman lab).

Equipment: Micro centrifuge (Qiagen), Water incubator (Fisher Scientific), Culture incubator (Sanyo), Shaker incubator (New Brunswick Scientific), Spectrometer (Bio-Rad).

Disposables: 1.5 ml microfuge tubes (USA Scientific), 15 ml falcon tissue culture tubes (BD Bioscience), 50 ml centrifuge tubes (Denville), Petri dishes (Fisher Scientific), 1.5 ml curvette (VWR).

In order to utilize the yeast system, we modified the *S. cerevisiae* strains original provided by Dr. Thomas Fowler (Southern Illinois University, Edwardsville) using the following yeast transformation protocol. We generated a strain YDX4, in which three genes in

pheromone pathway (*STE3*, *SST2*, and *FAR1*) were deleted to optimize signal output. The vector pTCFL1 containing a *FUS1-lacZ* fusion cassette was used to transform YDX4 to generate YDX10, in which the *lacZ* gene will be activated by pheromone response signals.

A. Protocol for Yeast transformation: Day 1: Inoculate a single yeast colony into a 5 ml YPD culture and incubate overnight (24 hr) with shaking at 30°C.

Day 2: Take out 120 µl overnight yeast culture and re-inoculate into 5 ml fresh YPD liquids and incubate for 5 hrs. After 5 hr incubation, centrifuge the culture at 2000 rpm for 3 min, and resuspend the pellet in 1 ml sterile deionized water (dH₂O) and transfer cells to 1.5 ml eppendorf tubes. Centrifuge again for 10 seconds fast spin, wash pellets twice with 1 ml dH₂O and wash once with 1 ml 0.1 M Lithium acetate (LiOAc). Resuspend washed pellets into 25 µl 0.1 M LiOAc. Add the following reagents to each tube: 200 µl 50% PEG (MW3640), 25 µl 1M LiOAc, 25 µl dH₂O, 5 µl 10mg/ml ssDNA, < 10 µl plasmid DNA. Mix each reaction gently and incubate at 30°C for 30 min. Transfer tubes to 42°C for 15 min. Centrifuge and resuspend pellets in 100 µl dH₂O and spread on selective medium, SD-Trp-Ura, for pTCFL1 and pPGK based vectors. Incubate at 30°C for 2–3 days.

Day 5: Streak out single colonies on a fresh SD-Trp-Ura plate. Typically at least three colonies should be streaked out for each transformation.

Yeast transformation is a routine technique and many modified protocols are available. We found the above protocol provided a simple method with consistent high transformation efficiency. Our experience with yeast transformation showed it is very important to not overgrow the culture after the second inoculation. Also always purify yeast colonies by re-streaking on selective medium to make sure only transformants expressing selective markers are obtained.

Upon the generation of the yeast strain, we first validated the sensitivity of the system by measuring the β-galactosidase activity (see below for protocol) for all three yeast strains, SDK45, SDK47, and YDX4. The supernatant from an overnight culture of W303-1A, which contains *a*-factor secreted by this *MATa* strain, was used as a ligand to activate the system. Strong induction of β-galactosidase activity was observed in all three strains background when native Ste3 was expressed, almost no signal was detected when the empty vector pPGK was introduced. These results indicate the system is working.

B. Protocol for measuring the beta-galactosidase activity in liquid cultures: Day 1: Start a 2 ml overnight culture in yeast dropout medium and let it grow at 30°C with shaking for about 24 hours.

Day 2: Centrifuge 1 ml of culture in a microfuge tube for about 2 second. Cell pellets were resuspended in 0.5 ml 1x Z-buffer (add 67 µl beta-mercaptoethanol, 19 µl 10% SDS, 5 ml 5x Z buffer, and 19.914 ml ddH₂O to make 25 ml of 1x Z-buffer) and vortexed for 1 min at room temperature. Add 25 µl chloroform and vortex for 5 min at room temperature. Add 100 µl CPRG solution (final concentration of CPRG is 4 mM) and incubate at 28°C until red color appears (8–20 min). Record the time of incubation. Put the sample on ice for 5 min to “stop” the reaction. Spin out debris for 5 min at full speed at 4°C and transfer 500 µl to cuvette and read O.D.₅₉₅ in a spectrometer. Prepare appropriate dilution for measurement if color is too intense. Also read O.D.₆₀₀ for the remaining 1 ml of overnight culture (usually needs 1:5 dilution). Calculate the enzyme activity using the following formula:

$$\beta\text{-galactosidase activity (Miller Units)} = 1000 \cdot (O.D._{595}) / ((\text{volume assayed}) \cdot \text{time} \cdot (O.D._{600}))$$
 . At least three replicates are needed for enzyme activity quantification because of the potential variation between different replications.

This is a simple protocol to quickly determine the β -galactosidase enzyme activity. It is important to measure the absorbance quickly to obtain representative results, because the color will increase over time even when tubes are kept on ice to “stop” the reaction.

2.2.2. Assessment of constitutive activity of Cpr2 in the yeast system—The establishment of a yeast system that can be used to quantify the activity of pheromone responsive pathway by measuring the β -galactosidase activity allowed us to test the expression and activity of Cpr2 in this system. The *CPR2* overexpression construct YPH58 was used to transform SD45, SDK47, and YDX4. The expression of *CPR2* was confirmed by Northern blot. In this experiment, pPGK empty vector was used as a negative control and a vector expressing native Ste3 from *S. cerevisiae* was used as the positive control. Yeast cells expressing *CPR2* and *FUS1-lacZ* were cultured on a yeast drop-out medium (SD-Trp-Ura) and β -galactosidase activities were measured following induction with different extracellular signals. Surprisingly, high β -galactosidase activity readouts were generated without exposing cells to putative ligands such as *C. neoformans* MFa or MFa synthetic pheromones (Hsueh et al., 2009) (Figure 2). Cpr2 induced high levels of β -galactosidase activity even in strain SDK45 background, which still has the wild-type *SST2* and *FAR1* alleles, and therefore, we have carried out the rest of the assays in strain SDK45 (Figure 2). The *S. cerevisiae* reporter strains SDK45, SDK47, and YDX10 are MAT α ; therefore, they produce only α factor, which should not act on GPCRs related to Ste3 (respond only to lipid-modified peptides).

The highly induced β -galactosidase activity in *CPR2*-expressing cells suggested that Cpr2 was either activated by a ubiquitous ligand present in the culture supernatants or that Cpr2 is a constitutively active GPCR that functions in a ligand-independent manner. The further identification of a single amino acid that is responsible for the enzyme activity without additional ligands confirms that the Cpr2 wild type form is a naturally-occurring, constitutively active receptor (Hsueh et al., 2009).

2.2.3 Identification of Leu²²² as the cause of constitutive activity of Cpr2—It is known that alteration of amino acid sequences in certain conserved positions in some GPCRs results in constitutively active mutants (CAMs). 90% of GPCRs contain a conserved proline in the sixth transmembrane domain (TM-VI), and substitution of this proline with leucine increases the ligand-independent activity of many GPCRs (Baldwin, 1993). CAMs have been generated using such point mutations in the pheromone receptors of *S. cerevisiae* (Ste2, Ste3) and *Schizosaccharomyces pombe* (Mam2) (Konopka et al., 1996; Ladds et al., 2005; Stefan et al., 1998). Surprisingly, this conserved proline is absent in the TM-VI of wild-type Cpr2, and instead, a leucine is substituted at this position Leu²²². To test the possibility that this leucine in TM-VI might be responsible for the constitutive activity of Cpr2, a construct (YPH59) expressing the *CPR2*^{L222P} allele was introduced in *S. cerevisiae* SDK45. As shown in Figure 2, ligand-independent induction of β -galactosidase activity was reduced to the basal level in cells expressing *CPR2*^{L222P}, compared to the empty vector control. This result provides evidence that Cpr2 activates the pheromone response pathway via an intrinsic constitutive activity instead of responding to a ubiquitous ligand present in the culture.

Because *CPR2* was overexpressed in the *S. cerevisiae* heterologous system, one possibility is that the overexpression might cause the constitutive activity of Cpr2. It has been reported that even some wild-type ligand-dependent GPCRs, a small fraction of the total receptor pool may exist in the active form in the absence of agonist; thus, simply overexpressing some GPCRs could elicit a constitutively active response (Milano et al., 1994). However, we do not think that overexpression alone is sufficient in the case of Cpr2-induced pheromone

signaling because overexpressing either the canonical pheromone receptor Ste3 α or the Cpr2^{L222P} allele did not activate the *FUS1-lacZ* reporter gene in *S. cerevisiae* (Figure 2).

Several other lines of evidence, including phenotypic analysis of *cpr2* mutants in *C. neoformans*, confirmed our conclusion. Overexpression of the Cpr2^{L222P} allele in the *C. neoformans ste3 α* mutant did not cause a hyperfilamentation phenotype as did of Cpr2 overexpression. Overexpression of Cpr2 in a *C. neoformans mfa 1, 2, 3* pheromoneless mutant still conferred a hyperfilamentous phenotype, eliminating the possible involvement of an autocrine-signaling loop. In protein-protein interaction assays using a split-ubiquitin yeast two-hybrid system, only the interactions of Cpr2^{L222P} with Gpa2 and Gpa3, but not of Cpr2 with these G proteins were detected, implying the interaction between wild type Cpr2 and the G α subunits could be transient due to its constitutive activity (Hsueh et al., 2009).

Although in many cases, including Cpr2, substitution of proline with leucine increases constitutive activity of GPCRs, not all GPCRs become constitutively active when this proline residue is missing or mutated. The Ste3 α receptor in *C. neoformans* is one such example, as it lacks a conserved proline in TM-VI but does not display any constitutive activity.

We have also tried to identify the potential inverse agonist agents for Cpr2 by testing the synthetic mating pheromones MF α and MF β , as well as a range of farnesol derivatives (pheromones are farnesylated) in the yeast heterologous expression system. None of these chemicals significantly altered the readout of β -galactosidase activity. Therefore, inverse agonists for Cpr2, if they exist, remain to be identified. Their existence is an intriguing hypothesis for future investigation.

2.2. 4. Limitation of the yeast heterologous expression system and

alternatives—Not all GPCRs can be expressed correctly, couple with *S. cerevisiae* Gpa1 and activate the downstream pheromone pathway. One example is Gpr4 in *C. neoformans*. We cloned *GPR4* in pPGK and expressed it in YDX10, but did not observed any pheromone-induced lacZ activity. So modifying the system, such as making chimeric G proteins, or chimeric GPCRs may expand the system for the analysis of other receptors that do not directly express well in yeast.

Actually, in most cases, modifications of Gpa1 or the GPCR itself are necessary to promote proper GPCR-G protein interaction and pathway activation. For example, most GPCRs couple to the C terminus of the G protein Gpa1, and one way to improve coupling between foreign receptors and Gpa1 without interfering Gpa1 activity is to generate a chimeric G α by replacing the Gpa1 C-terminal region with the corresponding protein of the foreign G α . Some studies indicate that replacing only 5 amino acids is sufficient to promote specific GPCR-G α coupling (Komatsuzaki et al., 1997; Kostenis et al., 1997; Montesana et al., 2002). We have constructed three chimeric G proteins where C-terminal 30 amino acids of *S. cerevisiae* Gpa1 was replaced by the corresponding C-terminal region of G α proteins (Gpa1, Gpa2, and Gpa3) in *C. neoformans*, respectively. These chimeric G proteins have been successfully expressed in a yeast strain with *STE3*, *SST2*, and *FAR1* all mutated (Xue et al., unpublished). Another approach that has been successfully applied is generating chimeric proteins by fusing adrenergic receptors and their cognate G α subunits (Bertin et al., 1994; Wise et al., 1997). However, this approach is not suitable for all receptors, suggesting the mechanisms for the pathway activation may be more complex.

Modifying the receptor itself is another way to improve foreign receptor expression and signal activation in yeast. Foreign GPCRs fused with the cytoplasmic domain of the Ste2 or Ste3 pheromone receptor serve to enforce coupling between receptor and Gpa1 (King et al.,

1990). This approach is particularly useful for GPCRs with unknown cognate G α subunits (Yin et al., 2004).

As an alternative, there are other systems available to serve similar purposes. In the fission yeast *S. pombe*, the GPCR Stm1 has been identified as a nutrient sensor. Overexpression of *STM1* promotes uncontrolled cell division of yeast cells, which triggers a severe growth defect and also conversion of diploid to haploid cells (Chung et al., 2003). This phenotype has been successfully utilized for high throughput screens of Stm1 inhibitors. Chemical compounds were applied to an *STM1* overexpression strain and inhibitors of Stm1 rescued the growth defect of the test strain (Chung et al., 2007). Because Stm1 interacts with the G protein Gpa2 to exert its effect on cell growth (Chung et al., 2003), this system can also be used to test heterologous GPCRs for their interactions with Gpa2 and screen for potential modulators.

3. Additional constitutively active receptors identified in fungi

Although no naturally-occurring constitutively active GPCR has been reported in ascomycetes, mutagenesis has been used to introduce mutant receptors with constitutive activity. The pheromone receptor Ste2 has been most thoroughly studied to identify amino acids important for function. Both site-directed mutagenesis and PCR-derived random mutagenesis approaches have been applied to select point mutations that cause the receptor to be constitutively active or dominantly negative (Konopka et al., 1996; Lee et al., 2006; Sommers et al., 2000). A number of sites in Ste2 have been identified as critical for ligand binding and protein function using these approaches. Also in the fission yeast *Schizosaccharomyces pombe*, a constitutively active mutant of P-factor receptor Mam2 (P262L) has also been reported (Ladds et al., 2005)

Many basidiomyceteous fungi, such as mushrooms, contain multiple sexes controlled by many different pheromone receptors. For example, in the mushroom *Coprinus cinereus*, the multiallelic B mating type genes encode a large family of seven-transmembrane domain receptors and CaaX-modified pheromones. According to results obtained using a yeast heterologous expression assay, the *C. cinereus* pheromone receptor acts as a G protein-coupled receptor and self-compatible mutations cause its constitutive activation (Olesnicki et al., 1999). In the laboratory, two TM-VI mutations of pheromone receptors in *Schizophyllum commune* have been identified to produce constitutive receptor activity (Tom Fowler, personal communication). Similar to Cpr2, many of these receptors are not located in the *MAT* locus. It is possible that naturally occurring constitutively active receptors may exist to regulate the pheromone sensing response in other fungi. Thus, the methods used to assess constitutive activity of Cpr2 will be beneficial for the studies of other potential constitutively active receptors and their mutants.

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Figure 1.

The yeast pheromone responsive pathway and generation of the yeast heterologous expression system. The pheromone receptor, Ste2 or Ste3, activates the G α protein Gpa1 by converting GDP-bound to GTP-bound and promoting the release of the G $\beta\gamma$ subunits (Ste4 and Ste18) from the heterotrimeric G protein complex. Ste4 in turn activates PAK kinase Ste20, and MAP kinase cascade including MAPKKK (Ste11), MAPKK (Ste7), and MAPK (Fus3). The activation of transcription factor Ste12 by Fus3 promotes mating. To generate a yeast heterologous system for Cpr2 using this signaling pathway, the pheromone receptor Ste3 is deleted to eliminate signal interference by endogenous receptor. The RGS protein Sst2 and the cyclin-dependent kinase inhibitor Far1 are deleted to optimize the signal readout. The promoter of the pheromone inducible gene *FUS1* is fused with a reporter gene, such as *lacZ* or *HIS3* to quantitatively measure the pheromone response signal. In the Cpr2 study, the *lacZ* gene was used.

Figure 2.

The constitutive activity of Cpr2 is dependent on the Leu²²² residue based on assays in a yeast heterologous expression system. Cpr2 is heterologously expressed in SDK45 carrying the *FUS1-lacZ* reporter. Cells expressing *S. cerevisiae* Ste3 treated with yeast **a**-cell supernatant served as a positive control. Cells containing the empty vector pPGK served as a negative control. Cells expressing Cpr2 or Cpr2^{L222P} were measured for the β-galactosidase activity without treatment of potential ligands. All results are based on at least three repeats. Error bars indicate standard deviations.

Table 1

Plasmids and strains used for Cpr2 assessment

Strains	Genotype	References
W303-1A	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100</i>	Fowler et al., 1999
W303-1B	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100</i>	Fowler et al., 1999
SDK45	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100 ste3::ADE2</i>	Fowler et al., 1999
SDK47	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100 ste3::ADE2 sst2::LEU2</i>	Fowler et al., 1999
YDX4	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100 ste3::ADE2 sst2::LEU2 far1::HYG</i>	This study
YDX10	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp-1 ura3-1 can1-100 ste3::ADE2 sst2::LEU2 far1::HYG FUS1-lacZ-TRP</i>	This study
Plasmids	Description	References
pTCFL1	<i>FUS1::lacZ TRP1 AmpR</i>	Chen and Kurjan, 1997
pPGK	Yeast overexpression vector, PGK promoter <i>URA3 AmpR</i>	Kang et al., 1990
pYH58	<i>pPGK::CPR2 cDNA URA3 AmpR</i>	Hsueh et al., 2009
pYH59	<i>pPGK::CPR2^{L222P} cDNA URA3 AmpR</i>	Hsueh et al., 2009