Variable Dependence of Signaling Output on Agonist Occupancy of Ste2p, a G Protein-coupled Receptor in Yeast*  

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We report here on the relationship between ligand binding and signaling responses in the yeast pheromone response pathway, a well characterized G protein-coupled receptor system. Responses to agonist (α-factor) by cells expressing widely varying numbers of receptors depend primarily on fractional occupancy, not the absolute number of agonist-bound receptors. Furthermore, the concentration of competitive antagonist required to inhibit α-factor-dependent signaling is more than 10-fold higher than predicted based on the known ligand affinities. Thus, responses to a particular number of agonist-bound receptors can vary greatly, depending on whether there are unoccupied or antagonist-bound receptors present on the same cell surface. This behavior does not appear to be due to pre-coupling of receptors to G protein or to the Sst2p regulator of G protein signaling. The results are consistent with a signaling response that is determined by the integration of positive signals from agonist-occupied receptors and inhibitory signals from unoccupied receptors, where the inhibitory signals can be diminished by antagonist binding.  

Understanding the relationship between receptor occupancy by ligands and signaling responses is of fundamental importance in predicting and modulating the behavior of signaling pathways. Modeling of receptor signaling often starts with the assumption that signaling output is a linear function of receptor occupancy by agonist or that output is mediated by direct interactions of activated receptors with downstream binding partners or enzymes. However, the underlying mechanisms regulating signaling outputs remain poorly defined. One of the best characterized classes of receptors is the G protein-coupled receptor (GPCR)3 superfamily composed of seven transmembrane helix receptors that can recognize and respond to diverse environmental stimuli. Consistent with their important physiological roles, GPCRs constitute a major class of drug targets (1, 2). Although GPCRs can act via multiple pathways, in most cases their primary mode of signal transduction involves the activation of cytoplasmic heterotrimeric G proteins, resulting in release of GDP, binding of GTP, and dissociation of the Gα subunit from the dimer of the Gβ and Gγ subunits. Either or both of the dissociated G protein components then activates various downstream effectors.  

A particularly well characterized GPCR signaling system is the yeast pheromone response pathway in which the peptide pheromones α-factor and a-factor bind to the cellular receptors Ste2p and Ste3p, respectively, triggering activation of a cytoplasmic heterotrimeric G protein that, in turn, activates an MAPK cascade leading to changes in transcription and cell morphology in preparation for mating. Although there is low sequence similarity between yeast receptors and mammalian receptors, the trimeric G proteins of these systems are very similar, and the systems appear to be highly homologous; in some cases, the components of the two systems exhibit interchangeable function (3–7). A regulator of G protein signaling (RGS), Sst2p, mediates down-regulation of yeast pheromone responsiveness by accelerating GTP hydrolysis in a fashion similar to the action of mammalian RGS proteins (8). The yeast system has been used to decipher basic mechanisms of GPCR signaling based on its genetic tractability and on extensive characterization of the limited number of participating cellular components (two types of GPCRs, only one type of which affects pheromone signaling and only one type of trimeric G protein in each haploid cell type). The relative simplicity of the yeast system has also led to its use as the basis for extensive quantitative and modeling studies (9–17).  

To fully understand signaling responses initiated by agonist-bound receptors, a quantitative relationship must be established between the number of occupied receptors and the magnitude of downstream signaling responses. For example, the extent of downstream signaling by β-adrenergic receptors could be modeled based on a hyperbolic relationship describing binding of agonist to receptors, direct activation of G proteins benz-2-oxa-1,3-diazol-4-yl; dTA, [des-Trp1,Ala3,Nle12]α-factor; dTH, [des-Trp1,des-His5,Nle12]α-factor; au, arbitrary fluorescence unit; d-Tyr9, [d-Tyr9,Nle12]α-factor.

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§ The abbreviations used are: GPCR, G protein-coupled receptor; RGS, regulator of G protein signaling; CEN, centromeric; Nle, norleucine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; dTA, [des-Trp1,Ala3,Nle12]α-factor; dTH, [des-Trp1,des-His5,Nle12]α-factor; au, arbitrary fluorescence unit; d-Tyr9, [d-Tyr9,Nle12]α-factor.
by receptors, and a second hyperbolic relationship describing the binding and activation of downstream effectors such as adenylate cyclase by activated G proteins (18, 19). Signaling in the yeast pheromone response pathway is characterized by a remarkable proportionality between receptor occupancy and pathway activation, measured either via G protein dissociation, MAPK activation, transcriptional induction of reporter genes, or cell cycle arrest (14, 20), indicating that signal output is a direct function of the number of agonist-occupied receptors at the cell surface. In this paradigm, signaling output in response to a given sub-saturating agonist concentration would be expected to depend on the number of receptors expressed at the cell surface, such that increasing the numbers of receptors would result in enhanced numbers of occupied receptors, enhancing signaling responses to low concentrations of agonist. However, responses to pheromone in yeast have been reported to be insensitive to changes in receptor expression over a wide range of expression levels (21–24). To further explore the relationship between receptor occupancy and signaling output, we examined the effects of systematic alterations of receptor number on pheromone responses in yeast by varying receptor expression levels and by evaluating the effects of mixing varying ratios of α-factor agonists and antagonists. We are unable to describe the signaling responses that we observed in terms of models in which output is determined by absolute numbers of agonist-bound receptors. Furthermore, we find that the discrepancies in these models are not readily explained by limitations imposed by the abundances of interacting components, including trimeric G proteins and the RGS protein Sst2p. The pheromone signaling pathway appears to provide a readout of the fractional occupancy of agonist bound to receptors, rather than to the absolute number of agonist-occupied receptors, suggesting that ligand-free receptors may act to inhibit signaling by ligand-occupied receptors.

**Results**

**Effect of Varying Receptor Expression Levels on Pheromone Responses**—To quantitatively examine the relationship between signaling output and numbers of receptors at the cell surface, we performed assays of α-factor-dependent induction of the pheromone-responsive FUS1-lacZ reporter in yeast strains expressing varying levels of full-length Ste2p receptors ranging from <0.2 times the level expressed from the normal chromosomal STE2 locus to ~9 times this level. Transcriptional induction of the FUS1-lacZ reporter has been reported to be linearly related to pheromone responses assayed at earlier stages of the pheromone response pathway (14, 20). Reproducible quantitation of responses to pheromone was facilitated by procedures for minimizing ligand adsorption and by performing side-by-side assays of the strains being compared (see “Experimental Procedures”). Levels of receptor expression at the cell surface of different yeast strains were determined based on saturation binding of the fluorescent α-factor analog [Lys7(NBD),Nle12]α-factor (Fig. 1). The values of \( K_d \) and \( B_{max} \) determined for different strains, shown in Table 1, are in approximate agreement with previous binding measurements (25, 26). However, direct measurement of cell surface-binding sites was not feasible for strains expressing receptors under control of the repressed GAL promoter in a chromosomal ste2Δ background, because levels of binding of fluorescent ligand were too low to be detected. Thus, an estimate of the upper limit on cell surface expression under these conditions (less than 20% of the normal chromosomally encoded Ste2p) was derived based on immunoblotting of Ste2p in whole-cell lysates from this strain (24).

Contrary to the predictions of models in which signaling responses depend on the total number of agonist-occupied receptors on a cell, yeast cells expressing large numbers of receptors exhibit similar or slightly reduced sensitivities to pheromone compared with cells expressing smaller numbers of receptors.

1) Cells expressing very low levels of receptors from a repressed GAL promoter exhibit a ~2-fold decrease in EC\(_{50}\).
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FIGURE 2. Signaling responses to α-factor in strains expressing varying levels of Ste2p. FUS1-lacZ induction in response to α-factor in strains expressing Ste2p from a repressed galactose-inducible plasmid (A4754) (A), a CEN plasmid (A4648) (B) and a multicopy plasmid (A4650) (C) is shown. Each panel shows signaling responses to α-factor of a reference strain expressing Ste2p solely from the normal chromosomal STE2 locus (A818). Data are presented as the mean ± S.E. for three independent transformants.

FIGURE 3. Signaling responses to α-factor in strains expressing truncated Ste2p. FUS1-lacZ induction in response to α-factor in strains expressing truncated Ste2p(A305–431) from a CEN plasmid (A4649) or a multicopy plasmid (A4651) is shown.

compared with cells expressing receptors from the normal chromosomal locus (Table 1 and Fig. 2A), contrary to the expectation that cells with low abundances of receptors would be insensitive to agonist. These low-expressing cells also exhibit an ~2-fold reduction in maximal signaling levels compared with cells expressing receptors from the normal chromosomal STE2, suggesting that the number of cell surface receptors in this strain is reduced below the threshold required for maximal output at saturating occupancy.

2) Cells expressing receptors from a CEN plasmid in a chromosomal ste2-Δ host exhibit EC_{50} values and maximal signaling levels that are similar to the values of these parameters in the strain expressing the chromosomal copy of STE2 alone (Table 1 and Fig. 2B).

3) Cells expressing STE2 from a multicopy plasmid are slightly (~2-fold) less sensitive to pheromone than cells with a single chromosomal STE2 gene (Table 1 and Fig. 2C) and exhibit maximal responses similar to those of the strain with only a chromosomal STE2 gene. The observed increase in EC_{50} upon increasing receptor expression is particularly notable when considering the fact that the overexpressed receptors exhibit a modest decrease in K_{d} for α-factor compared with receptors expressed solely from the normal chromosomal locus (Table 1).

Effects of Interacting Proteins on Signaling by Cells Expressing Varying Numbers of Receptors—One explanation for the lack of dependence of signaling responses on receptor expression levels could be that a select sub-population of receptors is responsible for signaling, such that expression of high levels of receptors that are not part of this population would be irrelevant to responses. Because some receptors, including Ste2p (27, 28), have been reported to form stable complexes with G proteins (29), a class of receptors that are pre-associated with G proteins would be a candidate for such a sub-population. Another candidate class of receptors could be those associated with the RGS protein Sst2p, which has also been reported to associate with Ste2p (30).

Because stable interactions of Ste2p with both the trimeric G protein and Sst2p are reported to occur through the C-terminal tail of the receptor (28, 30), one approach to examining such interactions is to test the effects of varying receptor expression levels on signaling by C-terminally truncated receptors. Such receptors retain full signaling capabilities, mediating hypersensitive responses to the α-factor that can be explained by their inability to undergo down-regulation via phosphorylation and internalization (22, 31). Thus, as shown in Table 1, strains expressing C-terminally truncated receptors are characterized by EC_{50} values for α-factor that are ~20-fold lower and B_{max} values for binding [Lys7(NBD),Nle12]α-factor that are ~6-fold higher compared with strains expressing similar constructs encoding full-length receptors. These differences are not due to enhanced ligand binding affinity, as the K_{d} values for binding of [Lys7(NBD),Nle12]α-factor to truncated receptors are 2–3-fold higher than those for similarly expressed full-length receptors.

Cells expressing truncated α-factor receptors, like those expressing full-length Ste2p, exhibit pheromone responses that are not strongly dependent on levels of receptor expression (Fig. 3). The EC_{50} value for cells expressing truncated receptors from a multicopy plasmid is ~1.4-fold higher than for cells expressing the truncated Ste2p from a CEN plasmid, contrary to the expectation that increased receptor expression should enhance sensitivity to agonist. This suggests that neither the C-terminal tail of the receptor nor interactions of the tail with cytoplasmic proteins such as the G protein or the RGS protein...
are required to render signaling independent of changes in receptor expression. The lack of a decrease in EC_{50} with increasing cell surface expression, together with the fact that the truncated receptors expressed from the CEN plasmid are only 2-fold more abundant than the full length, also indicates that the low EC_{50} values observed for truncated receptors are not due to high expression levels of these receptors.

As an additional way of investigating of the role of cytoplasmic proteins in maintaining signaling independent of receptor expression levels, we examined the effects of increasing the stoichiometric ratio of G proteins to receptors on signaling responses. Overexpression of trimeric G proteins, while maintaining the relative stoichiometry between subunits as constant, has the biggest effect on enhancing sensitivity to agonist in a strain expressing the subunits from a multicopy plasmid. An increase in expression of the RGS protein Sst2p plays a major role in modulating signaling responses, and because Sst2p had previously been implicated in modulating the effects of changes in receptor expression (based on assays of pheromone-dependent growth arrest) (23), we also investigated the effects of deleting or overexpressing SST2 on FUS1-lacZ induction in strains expressing different numbers of receptors. As expected (20, 32), deletion of SST2 enhanced sensitivity to α-factor in all tested strains, reducing the EC_{50} value of reporter induction by ~100-fold (Table 3 and Figs. 6 and 7). However, contrary to the previous reports, SST2-Δ strains expressing different levels of receptors exhibited only minor differences in maximal induction and EC_{50} (Fig. 7). Strains expressing STE2 from CEN and multicopy plasmids displayed EC_{50} values that were 2–3-fold higher than the strain with only a chromosomal STE2 gene. Another report based on halo assays of cell cycle arrest also found that expression of high levels of receptors in the absence of Sst2p does not affect pheromone sensitivity (30).

In contrast to a previous report that deletion of SST2 results in constitutive signaling even in the absence of receptor (33), we find that cells lacking the RGS protein display only low levels of basal FUS1-lacZ expression (Fig. 7). The basis for this difference is not known, but it could involve differences in strain backgrounds.

To further investigate whether a particular stoichiometric ratio of Sst2p to receptors mediates the insensitivity of signaling responses to changes in receptor number, we examined the effects of overexpressing Sst2p. This was accom-
plished using a CEN plasmid encoding SST2 under control of the ADH1 promoter (34). Successful overexpression of this protein was evident from immunoblotting (Fig. 6) and from the increased EC<sub>50</sub> values and reduced maximal responses of all strains containing the ADH1-SST2 construct (Fig. 8 and Table 3), as expected for overexpression of a protein with GTPase activating activity (35). We observed an increase in the EC<sub>50</sub> value for signaling as receptor expression increases from GAL-repressed to chromosomal to CEN plasmid and only a marginal /H11011 2-fold decrease in EC<sub>50</sub> in comparing multicopy to CEN plasmid-expressed receptors in the SST2-overexpressing strains. Thus, it seems unlikely that Sst2p is the major limiting factor that restricts the population of signaling-competent receptors.

Disparate effects on maximal signaling and sensitivity to pheromone were observed in evaluating the effects of Sst2p overexpression on yeast strains expressing different levels of Ste2p receptors (Table 3). Sst2p overexpression in cells expressing the lowest numbers of receptors (repressed GAL-STE2) led to the largest decrease in maximal signaling strength (/H11011 5-fold) but only a modest (/H11349 2-fold) increase in EC<sub>50</sub>. In contrast, Sst2p overexpression in cells expressing receptors from CEN or multicopy plasmids resulted in only ≈2-fold decreases in maximal signaling but larger (4–8-fold) increases in EC<sub>50</sub> values in cells expressing CEN or multicopy plasmid-encoded Ste2p. These effects can be explained in the following ways. 1) At low receptor expression levels and low agonist concentrations, the signaling output is primarily limited by the low like-

### TABLE 2

Binding and signaling parameters for strains overexpressing G proteins

| Full-length STE2 allele | G protein expression level<sup>a</sup> | Yeast strain | Binding to Lys<sup>7</sup>-NBD-α-factor<sup>b</sup> | FUS1-lacZ induction in response to α-factor<sup>c</sup> |
|------------------------|----------------------------------------|--------------|-----------------------------------------------|--------------------------------------------------|
| Chromosomal            | Chromosomal                            | A818         | ND<sup>d</sup>                                 | ND                                               |
| Repressed GAL1-STE2    | Overexpression                         | A4780        | ND                                              | 16 ± 2                                            |
|                         | Chromosomal                            | A4754        | ND                                              | 16 ± 2                                            |
|                         | Overexpression                         | A4779        | ND                                              | 16 ± 2                                            |
| CEN-STE2               | Chromosomal                            | A4648        | 7 ± 0.7                                         | 16 ± 2                                            |
|                         | Overexpression                         | A4781        | 5 ± 2                                           | 16 ± 2                                            |
| Multicopy STE2         | Chromosomal                            | A4650        | 6 ± 2                                           | 16 ± 2                                            |
|                         | Overexpression                         | A4782        | 5 ± 2                                           | 16 ± 2                                            |

<sup>a</sup> The strains overexpressing G proteins contain a multicopy plasmid with all three subunits of G proteins expressed under their respective native promoter.

<sup>b</sup> The relative B<sub>max</sub> = B<sub>max</sub> of the strain/B<sub>max</sub> of the strain A4648 expressing CEN-STE2 and chromosomally encoded G protein.

<sup>c</sup> The relative maximal induction = maximal induction of the strain/maximal induction of the strain expressing chromosomal Ste2p, chromosomal G protein; maximal induction for strain A818 (chromosomal STE2, chromosomal G protein) = 680 ± 32 au.

<sup>d</sup> ND means not determined.

FIGURE 5. Signaling responses to α-factor in strains overexpressing G proteins. FUS1-lacZ induction in response to α-factor binding in strains overexpressing G proteins and expressing Ste2p from a repressed galactose-inducible plasmid (A4779) (A), the chromosome (A4780) (B), a CEN plasmid (A4781) (C), or a multicopy plasmid (A4782) (D) is shown. Closed circles represent strains expressing G proteins from the chromosome, and open circles represent strains overexpressing all three subunits of G protein.
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TABLE 3

| Full-length STE2 allele | Sst2p expression level | Yeast strain | Binding to Lys7-NBD-α-factor | FUS1-lacZ induction in response to α-factor |
|-------------------------|------------------------|--------------|------------------------------|--------------------------------------------|
|                         |                        |              | Kd                          | EC50                                       |
|                         |                        |              | Relative Bmax               | Relative Maximal induction                  |
|                         |                        |              | max                         |                                            |
| Repressed GAL1-STE2     | Chromosomal           | A4754        | ND                          | 13 ± 1                                      |
|                         | Overexpression         | A4755        | ND                          | 23 ± 10                                    |
| Chromosomal sst2-∆      | A5259                 | 15 ± 11      | 1.3 ± 0.8                   | 0.06 ± 0.02                                |
| Chromosomal             | A818                  | 24 ± 22      | 1.3 ± 0.8                   | 0.13 ± 0.01                                |
| Overexpression          | A4757                 | 3 ± 0.3      | 2.5 ± 1                     | 0.14 ± 0.01                                |
| CEN-STE2               | A5260                 | 7 ± 0.8      | 5.8 ± 2                     | 325 ± 50                                   |
| sst2-∆                 | A4648                 | 5 ± 0.1      | 9 ± 4                       | 43 ± 8                                     |
| Overexpression          | A4744                 | 8 ± 0.7      | 12 ± 5                      | 156 ± 39                                   |
| Multicopy STE2          | A5261                 | ND           | ND                          | 0.8 ± 0.2                                  |
| Chromosomal             | A4650                 | 7 ± 0.1      | 9 ± 4                       | 0.9 ± 0.04                                 |
| Overexpression          | A4746                 | 8 ± 0.7      | 12 ± 5                      | 0.5 ± 0.02                                 |

a The strains overexpressing Sst2p have a CEN plasmid expressing Sst2p under the constitutive ADH promoter.
b The relative Bmax = Bmax of the strain/Bmax of the strain expressing chromosomal Ste2p, chromosomal Sst2p; Bmax for strain A818 (chromosomal Ste2p, chromosomal G protein) = 12 ± 5 nM; n = 3.
c The relative maximal induction for SST2- and Sst2 overexpressing strains is shown as the maximal induction of the strain/maximal induction of strain A818 expressing chromosomal Ste2p and chromosomal Sst2p. Maximal induction for sst2-∆ strains is shown as the maximal induction of the strain/maximal induction of sst2-∆ strain A5259 expressing chromosomal Ste2p.
d ND means not determined.

FIGURE 6. Western blots with antibodies against Sst2p (gift from Dr. Orna Resnekov, Molecular Sciences Institute) and glucose-6-phosphate dehydrogenase. A, strains overexpressing Sst2p were immunoblotted with anti-Sst2p. An sst2-∆ strain and a strain expressing wild-type amounts of Sst2p (from the chromosome) are shown for control. Sst2p runs at ~82 kDa. B, loading control using anti-glucose-6-phosphate dehydrogenase (G6PDH).

FIGURE 7. Signaling responses in strains lacking Sst2p. FUS1-lacZ induction in response to α-factor binding in sst2-∆ strains either containing a chromosomal deletion of STE2 (A5255) or expressing Ste2p from the normal chromosomal locus (A5259), a CEN plasmid (A5260), or a multicopy plasmid (A5261) is shown.

The likelihood of the sparse population of agonist-bound receptors encountering and activating G protein trimers and is, thus, relatively unaffected by changes in the GTPase activity brought about by Sst2p overexpression. 2) At intermediate levels of activated receptors (saturating agonist concentrations at low receptor expression levels or low agonist concentrations at higher receptor expression levels), activation of GTPase activity by overexpression of Sst2p has strong effects on signaling by the abundant activated G proteins. 3) At very high levels of activated receptors (high agonist and high expression levels), overexpression of Sst2p does not provide enough of a stoichiometric excess to strongly affect signaling output by the abundant activated G proteins.

Inhibition of Agonist Signaling by Antagonists—Several variant forms of α-factor with alterations in the N-terminal region of the pheromone peptide have been reported to act as antagonists toward the pheromone response pathway (Table 4). These include [des-Trp1,Ala3,Nle12]α-factor (dTA) (23, 36–39), [d-Tyr3,Nle12]α-factor (d-Tyr) (40), and [des-Trp1,des-His3,Nle12]α-factor (dTH) (40). All three of these compounds were able to compete effectively with [Lys7(NBD),Nle12]α-factor for binding to Ste2p receptors in cells expressing the receptors from CEN or multicopy plasmids as indicated by the measured IC50 values and the calculated Kd values shown in Table 4 (also refer to Fig. 9). All antagonists exhibited Kd values that were similar to the Kd value of the agonist for binding. To test whether the antagonists are associated with any partial agonist activity, we tested their abilities to activate the FUS1-lacZ reporter when present as the only ligand. None of the antago-
nists tested caused activation of the Ste2p receptors, even at concentrations as high as 5 nM (with the exception of a 2-fold enhancement over basal signaling elicited by dTA treatment of cells expressing Ste2p from a multicopy plasmid) (Fig. 10).

Previous studies have documented inhibition of signaling responses to α-factor by antagonists (36, 38). To quantitatively characterize this inhibition, we performed assays of FUS1-lacZ induction by α-factor in the presence of various concentrations of antagonists, calculating dose ratios according to the Schild formalism (41) as described under “Experimental Procedures” (Fig. 11). A Schild ratio or dose ratio, defined as the ratio of agonist concentrations required to elicit the same response in the presence and absence of the antagonist, was calculated for each antagonist. We calculated ratios associated with the EC50 for agonist in the presence and absence of antagonist by fitting levels of induction of the FUS1-lacZ reporter in response to varying concentrations of α-factor to a sigmoidal dose-response curve (Table 5).

For pure competitive antagonism, the dose ratio can be predicted based on the binding parameters of agonist (Table 1) and antagonist (Table 4). Specifically, addition of a concentration of antagonist [B] shifts the dose-response curve by \( \frac{1}{K_{d}(\text{antagonist})} \), where \( K_{d}(\text{antagonist}) \) refers to the dissociation constant for binding of the antagonist to Ste2p (41). Thus, the dose ratio depends solely on the concentration of the antagonist used and the \( K_{d} \) value of the antagonist. For strains expressing receptors from the chromosomal locus or from a repressed galactose-controlled promoter, where it is not possible to directly determine reliable inhibition constants for binding, we used the \( K_{d}(\text{antagonist}) \) values obtained from strains expressing Ste2p from a CEN plasmid.

A ~25–200-fold discrepancy is evident in comparing the measured dose ratios derived from signaling assays with the expected dose ratios calculated from the binding affinities (Table 5). This discrepancy exists at every receptor expression level and for all three antagonists tested. In view of the

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**TABLE 4**

| Ligand type | Name | Sequence | \( K_{i}(\text{CEN STE2})^{a} \) | IC50 (CEN STE2) | \( K_{i}(\text{multicopy STE2})^{b} \) | IC50 (multicopy STE2) |
|-------------|------|----------|-------------------------------|-----------------|--------------------------------|---------------------|
| Agonist     | α-Factor | WHWLQKPGQ\(P\text{Nle})Y | 12 ± 3 | 52 ± 11 | 14 ± 1 | 34 ± 3 |
| Antagonist  | dTA (des-Trp1,Ala3) | -HALQLKPGQ\(Nle)Y | 5 ± 2 | 23 ± 6 | 10 ± 4 | 24 ± 9 |
|             | n-[f]Tyr^c | WH(f)QLKPGQ\(Nle)Y | 6 ± 1 | 27 ± 4 | 25 ± 5 | 62 ± 13 |
|             | dTH (des-Trp1,des-His3) | -WLQLKPGQ\(Nle)Y | - | - | - | - |

^a This was performed in yeast strain A4648.
^b This was performed in yeast strain A4650.
^c Nle refers to norleucine.

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**FIGURE 8.** Signaling responses to α-factor in strains overexpressing Sst2p. FUS1-lacZ induction in response to α-factor binding in strains overexpressing Sst2p and expressing Ste2p from a repressed galactose-inducible plasmid (A4755) (A), the chromosome (A4757) (B), a CEN plasmid (A4744) (C), or a multicopy plasmid (A4746) (D) is shown. Closed circles represent strains expressing Sst2p from the chromosome, and open circles represent strains overexpressing Sst2p (under the ADH1 promoter).
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high levels of propagated errors in comparing the measured and expected ratios, it is difficult to determine whether the discrepancies in dose ratio measured for different strains and antagonists are significantly different from each other.

The Schild formalism is often presented as a plot of the logarithm of the dose ratio − 1 against the logarithm of antagonist concentration for a family of agonist-response curves measured in the presence of varying concentrations of antagonist. The hallmark of simple competitive antagonism in such plots is the presence of a linear relationship with a slope of 1 (42). Consistent with the discrepancies in dose ratios noted above, when we performed such an analysis, the resulting plots were not linear and did not exhibit slopes close to 1 (results not shown). A non-linear least squares analysis of a family of dose-response curves in the presence of different concentrations of antagonist (43) also failed to provide a useful fit to the families of dose-response curves.

One possible source of the discrepancy between expected and measured dose ratios could have been that the assay conditions used for measuring ligand binding are not identical to those used for signaling assays. The signaling assays are performed at 30 °C, in un-buffered media (which reaches a pH of ∼4.0 when cells grow to A600 ~ 1.0), whereas binding assays are generally performed in 20 mM acetate buffer at a pH of 4.6 (44). Furthermore, in performing binding assays, the cells are maintained at 0 °C until the sample is analyzed by flow cytometry, to limit internalization of ligand (25). The effects of these conditions were therefore examined to determine whether they could be contributing to the observed discrepancies. As shown in Table 6, it was possible to conduct saturation and competition binding assays using the fluorescent agonist [Lys7 (NBD),Nle12]α-factor under the conditions used in signaling assays (30 °C, in unbuffered media). The use of these conditions for binding did not significantly alter the Kd value for the labeled agonist or the IC50 value for competition binding of unlabeled antagonist. It did not prove to be feasible to conduct signaling assays under the same conditions used for binding, as the use of low temperatures and acidic buffer prevented induction of the FUS1-lacZ reporter.

Effects of Interacting Proteins on Antagonism—To determine whether interactions with the cytoplasmic G protein or the RGS protein Sst2p might play a role in modulating the inhibitory effects of antagonists on signaling, we examined the effects of antagonists on signaling responses of C-terminally truncated receptors that are expected to have reduced interactions with G proteins and Sst2p (28, 30). Saturation and competition binding assays revealed that the truncated receptors expressed from CEN and multicopy plasmids bind to agonists (Table 1) and antagonists (Table 7) with Kd and IC50 values similar to those for binding to strains similarly expressing full-length receptors. As shown in Fig. 12 and tabulated in Table 7, truncation does not remove the dose ratio discrepancy, and the amounts of antagonist required to inhibit signaling by truncated receptors are still much greater than would be expected based on the relative binding constants of agonist and antagonist.

One possible explanation for the high concentrations of antagonist required to inhibit α-factor signaling would be if signaling was mediated by a sub-population of receptors that are preferentially activated by agonist but resistant to antag-
onist, because of low affinity and/or high IC50 for antagonist. To test the possibility that such an agonist-selective subpopulation might consist of receptors that are stably pre-associated with G proteins, we examined the effects of over-expressing G protein subunits on dose ratios for antagonist action. Increasing G protein expression did not result in any decrease in the discrepancies between the expected and measured dose ratios for antagonist treatment (Fig. 13 and Table 8).

As a further test for the effect of stable receptor-G protein interactions, we examined the dose ratios for competitive antagonism in cells expressing receptors covalently fused to G protein α-subunits. A STE2-GPA1 fusion was expressed from a multicopy plasmid in an ste2-Δ, GPA1Δ host strain (Table 9 and

TABLE 5

Expected dose ratios (DRbind) and measured dose ratios (DRsig) for agonist/antagonist mixtures at all Ste2p expression levels

| Ligand | EC50 | Expected** ± S.E. | Measured*** ± S.E. | Expected/measured |
|--------|------|------------------|--------------------|-------------------|
| Glucose repressed GALI-STE2 (A4754) | | | | |
| α-Factor alone | 11 ± 3 | 1 | |
| α-Factor + 5 μM | 168 ± 51 | 940 ± 260 | 16 ± 6 | 60 |
| α-Factor + 5 μM dTA | 180 ± 77 | 430 ± 100 | 17 ± 9 | 25 |
| α-Factor + 5 μM dTH | 154 ± 16 | 830 ± 160 | 15 ± 4 | 57 |
| Chromosomal STE2 (A818) | | | | |
| α-Factor alone | 18 ± 4 | 1 | |
| α-Factor + 5 μM d-Tyr | 394 ± 133 | 940 ± 260 | 22 ± 9 | 43 |
| α-Factor + 5 μM dTA | 155 ± 20 | 430 ± 100 | 9 ± 2 | 50 |
| α-Factor + 5 μM dTH | 488 ± 91 | 830 ± 160 | 27 ± 7 | 30 |
| CEN-STE2 (A4648) | | | | |
| α-Factor alone | 28 ± 6 | 1 | |
| α-Factor + 5 μM d-Tyr | 133 ± 14 | 940 ± 260 | 5 ± 1 | 200 |
| α-Factor + 5 μM dTA | 80 ± 18 | 430 ± 100 | 3 ± 1 | 150 |
| α-Factor + 5 μM dTH | 187 ± 8 | 830 ± 160 | 7 ± 2 | 125 |
| Multicopy STE2 (A4650) | | | | |
| α-Factor alone | 57 ± 10 | 1 | |
| α-Factor + 5 μM d-Tyr | 209 ± 33 | 520 ± 190 | 4 ± 1 | 140 |
| α-Factor + 5 μM dTA | 170 ± 31 | 370 ± 40 | 3 ± 1 | 120 |
| α-Factor + 5 μM dTH | 340 ± 65 | 200 ± 45 | 6 ± 2 | 33 |

* Because the errors on the expected and measured dose ratios are high, the expected/measured dose ratios are not considered to be significantly different from each other. ** The expected dose ratio = DRbind = 1 + [agonist]/K<sub>i</sub>. *** The measured dose ratio = DRsig = EC<sub>50</sub> in the presence of antagonist/EC<sub>50</sub> in the absence of antagonist.
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**TABLE 6**

Evaluation of differences between binding and signaling assays

| pH values     | 3.5 | 4 ± 1 | 1 | 0.03 ± 0.02 | 50 ± 5 |
|---------------|-----|-------|---|-------------|-------|
|               | 4.0 | 7 ± 1 | 1 ± 0.05 | 0.01 ± 0.01 | 40 ± 1 |
|               | 4.6 | 6 ± 1 | 0.9 ± 0.06 | 0.02 ± 0.01 | 30 ± 9 |

| Binding condition | Acetate buffer (0 °C) | 12 ± 2 | 1 | 0.02 ± 0.01 | 15 ± 6 |
|                   | Media (0 °C) | 4 ± 0.5 | 1 ± 0.08 | 0.03 ± 0.01 | ND |
|                   | Media (30 °C) | 12 ± 0.6 | 3.5 ± 0.3 | 0.2 ± 0.03 | 6 ± 2 |

*au indicates arbitrary fluorescence units.

*ND means not determined.

*The increased dose ratio is a result of internalization of the agonist-bound receptor and transit of newly synthesized receptors to the cell surface at 30 °C.

**TABLE 7**

*Ki, IC50, and dose ratio values for antagonist binding to strains expressing truncated Ste2p

| STE2 allele       | Ligand type | Ki (for binding) | IC50 (for binding) | EC50 | Expected dose ratio | Measured dose ratio | Expected/observed dose ratio |
|-------------------|-------------|------------------|--------------------|------|---------------------|---------------------|---------------------------|
| CEN-STE2 (∆305–431) (A4649) | Agonist | 0.7 ± 0.2 | 3.5 ± 0.5 | 565 ± 40 | 6 ± 2 | 101 |
|                   | +5 µM DvTyr | 9 ± 0.6 | 21 ± 1 | 3.5 ± 0.5 | 565 ± 40 | 6 ± 2 | 101 |
|                   | +5 µM dTa | 8 ± 4 | 18 ± 8 | 1.1 ± 0.3 | 664 ± 306 | 2 ± 0.7 | 373 |
|                   | +5 µM dTH | 13 ± 2 | 30 ± 6 | 2.6 ± 0.6 | 391 ± 74 | 4 ± 1.8 | 95 |
| Multicopy STE2 (∆305–431) (A4651) | Agonist | 1.3 ± 0.3 | 4 ± 0.8 | 237 ± 122 | 3 ± 1 | 78 |
|                   | +5 µM DvTyr | 22 ± 11 | 42 ± 21 | 4 ± 0.8 | 237 ± 122 | 3 ± 1 | 78 |
|                   | +5 µM dTa | 31 ± 7 | 60 ± 13 | 5 ± 1 | 163 ± 36 | 4 ± 1 | 47 |
|                   | +5 µM dTH | 28 ± 3 | 53 ± 4 | 7 ± 1 | 183 ± 18 | 5 ± 1 | 35 |

*The expected dose ratio = DRbind = 1 + [agonist]/Ki.

*The measured dose ratio = DRsig = EC50 in the presence of antagonist/EC50 in the absence of antagonist.

To optimize signaling function, the fusion consisted of a partially C-terminally truncated version of the receptor attached at its C terminus to Gpa1p, as described previously (24, 45). Cells expressing the STE2-GPA1 fusions as their only pheromone receptors exhibited dose ratios for antagonist that were similar to those for cells expressing normal complements of unfused receptors and G proteins. Thus, it is unlikely that the discrepancies in dose ratio can be explained by a sub-population of G protein-associated receptors that are primarily responsible for agonist-dependent responses but insensitive to antagonist.

To examine the possibility that association of Sst2p with Ste2p is involved in limiting inhibition of signaling by antagonist, we determined the dose ratios for antagonists in yeast strains overexpressing Sst2p (Table 10 and Fig. 15). (A low level of antagonist (3 µM) was used in this assay, compared with other dose-response experiments, because of the relative insensitivity of Sst2p-overexpressing strains to agonist.) Regardless of receptor expression levels, overexpression of Sst2p failed to alleviate the discrepancies between predicted and measured dose ratios. Thus, association of receptors with limited numbers of Sst2p proteins does not seem to be responsible for the discrepancies. In addition, a strain overexpressing both Sst2p and G proteins with chromosomally expressed receptors also failed to show an increase in the ability of antagonist to inhibit agonist signaling better (data not shown).

**Discussion**

We examined the relationship between receptor occupancy and signaling in the G protein-coupled pheromone response pathway of the yeast *Saccharomyces cerevisiae* in the following three ways: 1) incubating cells expressing a set number of receptors with varying concentrations of α-factor; 2) varying the number of cell surface receptors expressed on each cell; and 3) using competitive antagonists of α-factor to vary the number of sites available for agonist binding.

Cells expressing any particular number of receptors exhibit a striking linear correlation between signaling output (monitored by FUS1-lacZ reporter induction) and receptor occupancy predicted from binding isotherms. It has previously been reported that this proportionality of response is maintained at stages of the signal transduction pathway ranging from G protein activation through MAPK activation to transcriptional induction and cell cycle arrest (14, 20). This coincidence of normalized binding and response curves is consistent with paradigms established in mathematical treatments of receptor dose-response relationships (46) in which signaling output is determined by the total number of receptors occupied by agonist at any given time.

However, the results that we have obtained by varying receptor expression levels and by mixing agonist with competitive antagonist are not compatible with models in which signaling output is a simple function of the total number of agonist-occupied receptors. In agreement with previous reports (21, 22, 24), we find that cells expressing different numbers of total receptors at the cell surface require agonist binding to different absolute number of receptors to elicit the same response. In cells expressing low numbers of receptors, a small number of agonist-occupied receptors can elicit a strong response, whereas cells expressing higher numbers of receptors require...
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higher numbers of agonist-bound receptors to achieve the same response. Another way of viewing this is that the presence of a large number of unoccupied receptors in the higher expressing cells appears to inhibit signaling by the same number of agonist-occupied receptors that would give strong signaling in the lower-expressing cells. In fact, signaling responses to mixes of increasing concentrations of agonist and 5 μM D-Tyr³ (open circles), 5 μM dTA (open square), or 5 μM dTH (closed squares) are shown for each Ste2p expression level.

We also observed apparent variability in signaling responses elicited by a given number of agonist-occupied receptors in cells treated with mixes of agonist and antagonist. This was manifested as robust signaling responses to agonist in the presence of large excesses of three different antagonists that would each be expected to drastically reduce agonist binding. Because we confirm that agonist and antagonist do effectively compete for the same sites, this implies that the magnitude of signaling response per agonist-occupied receptor is actually enhanced in the presence of antagonist. As expected for competitive antagonists, the presence of the /H9251-factor antagonists results in a rightward shift of the dose-response curve while maintaining, at the highest agonist concentrations, the same maximal signaling responses seen in the absence of antagonist (Fig. 11). However, the magnitudes of the rightward shifts were considerably smaller than would be expected based on the known $K_i$ values of these compounds for inhibiting α-factor binding to receptors. Such large discrepancies were observed from the lowest to the

FIGURE 12. Signaling responses to agonist/antagonist mixtures in strains expressing truncated Ste2p. FUS1-lacZ inductions from strains expressing truncated Ste2p(³305–431) from a CEN plasmid (A4649) (A) or a multicopy plasmid (A4651) (B) are shown. Responses to agonist alone are shown by filled circles. Signaling responses to mixes of increasing concentrations of agonist and 5 μM D-Tyr³ (open circles), 5 μM dTA (open square), or 5 μM dTH (closed squares) are shown for each Ste2p expression level.

FIGURE 13. Signaling responses to agonist/antagonist mixtures in strains overexpressing G proteins (see also Table 8). FUS1-lacZ induction in strains overexpressing G proteins and varying levels of receptor from a repressed galactose-inducible plasmid (A4779) (A), a chromosome (A4780) (B), a CEN plasmid (A4781) (C), or a multicopy plasmid (A4782) (D). Responses to agonist alone are shown by filled circles. Signaling responses to mixes of increasing concentrations of agonist and 5 μM D-Tyr³ (open circles), 5 μM dTA (open square), or 5 μM dTH (closed squares) are shown for each Ste2p expression level.
high levels of receptor expression, indicating that this inefficiency in inhibiting agonist-dependent signaling does not depend on the presence of excess receptors.

Some signaling systems operate under conditions where only a fraction of the cellular complement of receptors needs to be occupied by agonist to elicit a maximal signaling response (46, 47). In this paradigm, the population of receptors in excess of what is required for maximal signaling output is referred to as “spare” receptors. Overexpression of receptors in recombinant systems provides a way of generating spare receptors, as verified for \( \beta_{2} \)-adrenergic receptors (48). However, overexpression of receptors in the yeast pheromone response pathway does not result in the expected hallmarks of excess spare receptors, such as a predicted mismatch between the dissociation constant for agonist binding and the EC\textsubscript{50} for signaling or deviations from sigmoidal shape (and Hill coefficient of unity) of dose-response curves (49). Furthermore, the signaling responses that we observe in the presence of increasing levels of Ste2p receptor expression levels do not exhibit the decrease in EC\textsubscript{50} that is expected for a system containing a homogeneous population of receptors present in greater numbers than needed to elicit a maximal signaling response. In fact, in comparing cells expressing GAL-repressed STE2 with cells expressing more than 40-fold receptor from a multicopy plasmid, the higher expression of cells exhibit a nearly 4-fold greater EC\textsubscript{50} value for \( \alpha \)-factor.

In contrast to the above, the signaling response of one particular set of yeast strains that we examined, those expressing C-terminally truncated Ste2p receptors, provided clear evidence for spare receptors, because the EC\textsubscript{50} value that we report for these cells is \( \sim 8–15 \) times lower than the \( K_a \) values measured for the same strains. However, even in these strains, dose-response curves do not exhibit the characteristic non-sigmoidal behavior expected for spare receptors (49), and expressing increased numbers of receptors does not decrease the EC\textsubscript{50} value.

Taken together, these results demonstrate that the relationship between signaling output and the number of agonist-occupied receptors is context-dependent and not a direct func-

### TABLE 8

| STE2 allele | Antagonist | \( K_a \) (for binding) | IC\textsubscript{50} (for binding) | EC\textsubscript{50} | Expected dose ratio\(^a\) | Measured dose ratio\(^b\) | Expected/observed dose ratio |
|-------------|------------|-------------------------|-------------------------------|-------------------|--------------------------|---------------------------|-----------------------------|
| Repressed GALI-Ste2p (A4779) | Agonist | 26 ± 12 | 100 ± 16 | 720 ± 290 | 4 ± 2 | 180 |
| | \( +5 \mu\text{M} \text{DTyr}^3 \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) |
| Chromosomal (A4780) | Agonist | 40 ± 16 | 102 ± 18 | 720 ± 290 | 3 ± 1 | 275 |
| | \( +5 \mu\text{M} \text{DTyr}^3 \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) |
| CEN-STE2 (A4781) | Agonist | 18 ± 2 | 62 ± 12 | 350 ± 210 | 2 ± 0.8 | 220 |
| | \( +5 \mu\text{M} \text{DTyr}^3 \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) |
| Multicopy STE2 (A4872) | Agonist | 47 ± 17 | 51 ± 6 | 473 ± 165 | 1 ± 0.4 | 435 |
| | \( +5 \mu\text{M} \text{DTyr}^3 \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) |

\(^a\) The expected dose ratio = \( DR_{\text{expected}} = 1 + \frac{[\text{agonist}]}{K_a} \).

\(^b\) The measured dose ratio = \( DR_{\text{measured}} = \frac{EC_{\text{50} \text{ in presence of antagonist}}}{EC_{\text{50} \text{ in absence of antagonist}}} \).

### TABLE 9

| STE2 allele | Ligand type | \( K_a \) (for binding) | IC\textsubscript{50} (for binding) | EC\textsubscript{50} | Expected dose ratio\(^a\) | Measured dose ratio\(^b\) | Expected/observed dose ratio |
|-------------|-------------|-------------------------|-------------------------------|-------------------|--------------------------|---------------------------|-----------------------------|
| Ste2p-Δ369–431-G\( \alpha \) fusion (A4881) | Agonist | 24 ± 3 | 348 ± 146 | 823 ± 307 | 14 ± 6 | 58 |
| | \( +5 \mu\text{M} \text{DTyr}^3 \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) | \( +5 \mu\text{M} \text{dTA} \) | \( +5 \mu\text{M} \text{dTH} \) |

\(^a\) The dissociation constant for (Lys\( ^{\text{NBD}} \),Nle\( ^{\text{NBD}} \))-factor binding to Ste2p-Δ369–431-G\( \alpha \) fusion (strain A4881) was 5 ± 2 nM.

\(^b\) The measured dose ratio = \( DR_{\text{measured}} = 1 + \frac{[\text{agonist}]}{K_a} \).

\(^c\) The measured dose ratio = \( DR_{\text{measured}} = EC_{\text{50} \text{ in presence of antagonist}}/EC_{\text{50} \text{ in absence of antagonist}} \).
tion of the absolute number of agonist-occupied receptors on the cell surface. Any explanation for this context dependence must directly involve the Ste2p receptors, because the relevant experimental manipulations consisted of changing Ste2p expression levels and treating cells with different combinations of extracellular Ste2p-directed ligands. We consider the following alternative mechanisms to explain these results,

1) A Specialized Sub-population of Receptors That Is Responsible for Pheromone Responses—If signaling is mediated by a special sub-population of receptors, then variations in the levels of expression of receptors that are not part of the sub-population would not affect signaling output (50, 51). The active sub-population might also have lower affinities for antagonist than the overall population, providing a basis for the weaker-than-expected competitive inhibition observed for the antagonists. Such a sub-population of sites could result from stable pre-association of receptors with a limiting cellular component, such as the trimeric G protein or an RGS protein. However, to date, we have been not been able to detect any sub-population of active receptors or provide any evidence for regulation of signaling by stable receptor-G protein or receptor-RGS complexes. (i) In particular, assays of binding affinities of receptors for agonist and antagonist can be readily fit by a simple population of sites with uniform affinities for ligands. However, a sub-population of receptors would be difficult to detect if the numbers of receptors involved were small or the binding affinities of the sub-population were not very different from the overall population. (ii) The effects of varying receptor expression level and the anomalous insensitivity of receptors to inhibition by antagonist that we observe are preserved upon C-terminal truncation of Ste2p receptors, removing reported sites of stable association with G protein and the RGS protein Sst2p (28, 30). (iii) Neither overexpression of all three subunits of the G protein nor deletion of the RGS protein, Sst2p, nor overexpression of Sst2p enhanced the dependence of signaling responses on receptor expression levels. This result is in contrast to some (23) but not all (30) previous reports. Overexpression of Sst2p or G protein subunits also failed to promote inhibition of agonist-dependent signaling by excess antagonists. (iv) Covalent fusion of receptors to G protein did not substantially affect the inability of antagonists to effectively inhibit signaling.

Deletion of SST2 has also been reported to confer on Ste2p-expressing cells the ability to respond to ligands that behave as antagonists toward SST2+ cells expressing normal receptors (23, 36). In view of the extreme insensitivity of SST2+ cells to high concentrations of α-factor antagonists that we report here (Fig. 10), the acquisition of signaling responses to antagonists is unlikely to be a simple result of the hypersensitivity of sst2− cells. (Deletion of SST2 confers an ~10-fold increase in pheromone sensitivity in our strains, yet a concentration of antagonist more than 100-fold above the measured dissociation constant fails to elicit a significant signaling response.) This

| STE2 allele | Antagonist | $K_d$ (for binding) | $IC_{50}$ (for binding) | EC$_{50}$ | Expected dose ratio | Measured dose ratio | Expected/observed dose ratio |
|------------|------------|---------------------|-------------------------|---------|-------------------|-----------------|-----------------------------|
| Chromosomal (A4757) | Agonist | $+3 \mu M$ D-Tyr$^3$ | 46 ± 20 | $850 \pm 65$ | $338 \pm 108$ | 19 ± 8 | 18 |
| | | $+3 \mu M$ dTA | 425 ± 12 | $333 \pm 62$ | 9 ± 4 | 36 |
| | | $+3 \mu M$ DTH | 2350 ± 1340 | 174 ± 48 | 51 ± 36 | 3 |
| CEN-STE2 (A4744) | Agonist | $+5 \mu M$ D-Tyr$^3$ | 9 ± 3 | 22 ± 7 | ND | ND | ND |
| | | $+5 \mu M$ dTA | 22 ± 7 | 22 ± 3 | ND | ND | ND |
| | | $+5 \mu M$ DTH | 17 ± 5 | 43 ± 11 | ND | ND | ND |
| Multicopy STE2 (A4746) | Agonist | $+5 \mu M$ D-Tyr$^3$ | 8 ± 2 | 17 ± 5 | ND | ND | ND |
| | | $+5 \mu M$ dTA | 22 ± 7 | 50 ± 14 | ND | ND | ND |
| | | $+5 \mu M$ DTH | 25 ± 6 | 56 ± 12 | ND | ND | ND |

$^a$ The expected dose ratio = $DR_{bind} = 1 + \text{[agonist]}/K_d$.

$^b$ The measured dose ratio = $DR_{sig} = EC_{50}$ in the presence of antagonist/EC$_{50}$ in the absence of antagonist.

$^c$ The measured dose ratios for the strain expressing chromosomal Ste2p were calculated with 3 μM antagonists instead of 5 μM, because we were unable to saturate downstream responses with strains overexpressing Sst2p with mixtures of agonist and antagonist. The expected dose ratios were also calculated for 3 μM concentrations.

$^d$ ND means not determined.
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supports the interesting idea that one or more functions of Sst2p are directly tied to the ability of Ste2p receptors to discriminate between agonists and antagonists. However, these signaling responses to antagonists would severely complicate any attempt to examine competition between agonist and antagonist in sst2Δ strains.

Although a relevant sub-population of receptors could be defined by interactions with a cellular protein other than the G protein or Sst2p, extensive classical mutational screening (52–55) as well as exhaustive testing of yeast deletion and repressible-promoter collections (56–58) have yet to identify likely novel candidates acting directly through interactions with the receptor.

2) A Regulatory System That Monitors Receptor Expression Levels in Order to Respond to Fractional Receptor Occupancy—

A direct way to provide signaling responses that report on fractional occupancy independent of total receptor expression levels would be for the signaling output to actually be the result of integrating a positive signal from agonist-occupied receptors and a negative signal from receptors that are not bound to agonist. This provides an intrinsic mechanism explaining the observed independence from receptor expression levels, but it also has the attractive theoretical property of rendering signaling responses independent of cell-to-cell fluctuations in receptor levels that are known to occur for production of many proteins due to inherent noise in transcription, translation, and intracellular trafficking systems in yeast (59). Distributions of cells with variations in the numbers of surface-exposed Ste2p receptors are readily observable in flow cytometric histograms of the overall fluorescence of cells with bound fluorescent ligands (25). The lack of dependence of the signaling potency of α-factor on receptor expression levels provides a way of maintaining an optimal dose-response alignment (14, 20) that could maintain maturing capabilities under varying growth or environmental conditions affecting receptor expression levels.

Several previous experimental results demonstrate that signal transduction by yeast pheromone receptors includes both activating and inhibitory aspects, as follows: (i) Deletion of the gene encoding the Ste2p receptor in some strains leads to an elevation of basal levels of signaling in the absence of α-factor (33, 38). This suggests that unactivated receptors may be capable of inhibiting the rate of spontaneous nucleotide exchange on the G protein. (ii) Signaling by constitutively active and hypersensitive mutations of STE2 is suppressed by co-expression of normal receptors in the same cells (22, 23, 31, 38, 60, 61), and the extent of this suppression is dependent on the relative expression levels of the normal and mutant receptors (38). (iii) A common class of mutations in the Ste2p receptor results in dominant negative effects, suppressing signaling by co-expressed normal receptors (21, 24, 28, 62). These mutants may have lost their capacity to initiate positive signaling while retaining an intact inhibitory component of their function. The dominant inhibitory action of these alleles generally requires that they be overexpressed relative to co-expressed normal receptors.

The mechanisms underlying the inhibitory behaviors of Ste2p are not clear. A first possibility is that inactive receptors can sequester G protein in an inactive state (21, 28, 62). However, this seems unlikely as an explanation for the current observations, because the signaling remains independent of receptor expression even for C-terminally truncated receptors, under conditions where G proteins are overexpressed, and at very low receptor expression levels. A second possibility is that inhibitory behavior is mediated by co-oligomerization of active (agonist-bound) and unactivated (ligand-free) receptors such that the inactive receptors directly inhibit activation of co-oligomerized agonist-bound receptors (see below for a fuller discussion). A third possibility is that unactivated receptors participate in direct interactions with G proteins that inhibit nucleotide exchange. Such inhibitory interactions could block both low basal levels of G protein activation and activating responses of agonist-occupied receptors.

The existence of inhibitory effects of unoccupied receptors could provide an explanation of the unexpected inefficiency of antagonist in inhibiting agonist-dependent signaling if antagonist binding diminishes the inhibitory effects on signaling by unoccupied receptors. This would enhance the signaling output of cells with a mixture of agonist- and antagonist-bound receptors compared with cells with a mixture of agonist-bound and unoccupied receptors. The possibility that antagonist binding can affect receptor behavior in a manner that goes beyond simply competitively inhibiting agonist binding is supported by observations that binding of an α-factor antagonist induces a conformation of Ste2p that is distinct from the agonist-bound or unliganded states (63) and by identification of a class of receptor mutations that allow activation of signaling responses by these nominally antagonistic compounds (38–40, 64, 65).

3) Allosteric Effects of α-Factor Antagonists on Ste2p Receptors—

The fact that a given number of agonist-bound receptors apparently elicits more robust signaling in the presence of antagonist than in the absence of competing ligand raises the possibility that the antagonist is acting allosterically at a second ligand-binding site on agonist-bound receptors to enhance signaling responses. To date, we have not been able to obtain evidence for such allosteric binding, either by saturation binding or competition experiments; however, the existence of a second site, either with low affinity or with an affinity similar to that of the normal orthosteric ligand-binding site, cannot be ruled out. The possibility of a second site is reinforced by the existence of certain α-factor analogs, known as synergists, that enhance Ste2p signaling but only in the presence of a normal agonist (36, 64). Also, a small antibiotic, novobiocin, is capable of activating Ste2p as well as variant forms of Ste2p that cannot be activated by α-factor (66).

4) Effects of Receptor Oligomerization—

There is considerable evidence that GPCRs exist in cells as oligomers (67), even though purified receptors are capable of functioning as monomers (68, 69). Oligomerization of yeast Ste2p receptors in cells has been demonstrated using fluorescence resonance energy transfer (70, 71), bioluminescence resonance energy transfer (26), co-immunoprecipitation (72), and disulfide cross-linking (73), although the size of the oligomer remains uncertain. Functional interactions among co-oligomerized receptors could provide an explanation for some of the unexpected signaling behaviors that we have observed, for example, if unoccupied receptors exert inhibitory actions on co-oligomerized agonist-
bound receptors. Furthermore, antagonist-bound receptors could enhance signaling by co-oligomerized agonist-bound receptors or block inhibitory interactions among co-oligomerized receptors.

If agonist binding to only one monomer of an oligomer is sufficient to elicit the full signaling response by the oligomer, then signaling by oligomers consisting of monomers bound to mixtures of agonist and antagonist could provide an explanation for higher-than-expected levels of agonist signaling when antagonist is present. However, simulations of this type of behavior that we have performed in non-cooperative oligomers of different sizes (results not shown; similar to the treatment of Marunaka et al. (74)) do not yield large enough changes in dose ratio to account for the discrepancies between expected and measured Fus1-lacZ responses that we observe for the tested antagonists. Such large discrepancies in dose ratios could, in fact, be due to cooperative interactions among ligand-bound receptors, such as the enhancement of response to agonist by one receptor in an oligomer brought about by binding of antagonist to a different component of the oligomer. Cooperative interactions among oligomerized GPCRs have been reported in some GPCR signaling systems (75), including enhancement of responses to dopamine agonist by co-oligomerized dopamine D2 receptors bound to an inverse agonist (76). Although negative cooperativity has been invoked as an explanation for dominant negative effects of mutant Ste2p receptors (24, 26), no evidence of positive cooperative interactions related to antagonist binding is available for Ste2p receptors.

The yeast pheromone response pathway is one of the best characterized GPCR signaling pathways based on its limited set of participating cellular components, on the extensive available database of results of genetic manipulation of these components, and on the well established quantitative assays of signaling outputs in cells. This pathway has thus served as the basis for efforts to quantitatively model and alter signaling behaviors (9–17). Our results point out unexpected complexity in the relationship between receptor occupancy and signaling output. In particular, signaling output per agonist-bound receptor decreases as the number of receptors at the cell surface increases and increases upon antagonist binding to receptors that are not occupied by agonist. Some mammalian GPCR signaling systems have been reported to exhibit decreases in EC_{50} values commensurate with expectations based on conventional models of spare receptors (48, 77–80), but other systems in which the number of cell surface receptors can be varied exhibit little change in EC_{50} values with varying receptor densities (78, 81–84). These findings, as well as the possible importance of inhibitory signaling by unoccupied receptors, will need to be considered in future efforts to obtain a quantitative understanding of signaling responses in yeast and mammalian systems.

**Experimental Procedures**

**Strains and Plasmids**—Table 11 lists the strains and plasmids used in this work. All transformations were performed using a modified one-step PEG/LiAc protocol according to Ref. 85, with the heat shock step performed at 42 °C instead of 45 °C. To minimize homologous recombination, all strains containing two plasmids were transformed sequentially. Strains expressing only one protein from a plasmid also contained an empty URA3 vector (pMD228) (21) or an empty LEU2 vector (yEpLac181) (86) to allow growth in the same media as strains expressing proteins from two plasmids.

pMD730 contains Ste2p under control of the GAL1 promoter (24); pMD1145 contains Ste2p in a CEN plasmid (25), and pMD240 is a multicopy plasmid expressing Ste2p (21). A tail-less version of Ste2p, truncated after residue 304 (Δ305–431), is expressed using pMD240 (21). To construct a plasmid overexpressing Sst2p under the ADH1 promoter with a LEU2 marker, pRS315-EV LEU2 (gift from Dr. Henrik Dohlman, University of North Carolina) (87) was digested with PvuI and ligated to PvuI-digested pRS316-promoter (24); pMD1231 (multicopy plasmid) (25). Multicopy STE2 plasmid (25). Multicopy STE2 plasmid (25).

| Strain no. | URA3 plasmid | LEU2 plasmid | Host background | Notes | STE2 allele |
|------------|--------------|--------------|----------------|-------|-------------|
| A4754      | pMD730       | pMD284       | A232           |       | GAL1-STE2   |
| A4718      | pMD228       | pMD284       | A232           |       | CEN-STE2    |
| A4648      | pMD1145      | pMD284       | A232           |       | Multicopy STE2 |
| A4650      | pMD240       | pMD284       | A232           |       | Multicopy STE2 |
| A4755      | pMD730       | pMD2405      | A232           |       | Sst2p overexpression GAL1-STE2 |
| A4757      | pMD228       | pMD2405      | A232           |       | Chromosomal |
| A4746      | pMD1145      | pMD2405      | A232           |       | Sst2p overexpression CEN-STE2 |
| A4746      | pMD240       | pMD2405      | A232           |       | Multicopy STE2 |
| A4779      | pMD730       | pMD854       | A232           |       | G protein overexpression GAL1-STE2 |
| A4780      | pMD228       | pMD854       | A230           |       | Chromosomal |
| A4781      | pMD1145      | pMD854       | A232           |       | G protein overexpression CEN-STE2 |
| A4782      | pMD240       | pMD854       | A232           |       | Multicopy STE2 |
| A4649      | pMD1231      | pMD284       | A232           |       | Truncated Ste2p (Δ305–431) CEN-STE2 |
| A4651      | pMD1422      | pMD284       | A232           |       | Multicopy STE2 |
| A4881      | pMD2445      | pMD284       | A232           |       | Ste2p truncated at residue 369 Multicopy STE2 |
| A4882      | pMD2515      | pMD284       | A232           |       | Ste2p truncated at residue 369 (Δ369–431)-Ga fusion Multicopy STE2 |
| A4810      | pMD746       | pMD854       | A230           |       | Sst2p and G protein O/E Chromosomal |
| A5255      | pMD559       | A1110         |                |       | sst2-Δ      |
| A5259      | pMD559       | A1111         |                |       | sst2-Δ      |
| A5260      | pMD149       | A1111         |                |       | sst2-Δ      |
| A5261      | pMD240       | A1111         |                |       | Chromosomal + CEN-STE2 |
|            |              |              |                |       | Chromosomal + multicopy STE2 |
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structured by digesting pMD739 (24) with KpnI and BamHI and ligating to KpnI-BamHI-cut pMD1055 (25), creating pMD2279. pMD854, a multicopy plasmid expressing all three G protein subunits under their own promoters, was constructed by inserting a KpnI-flanked STE18 PCR product into the KpnI site in pMD327, which contains the GPA1 and STE4 genes (21).

To express a Ste2p-Gpa1p fusion protein, pMD2445, a multicopy plasmid encoding the receptor truncated after residue 369 (Δ369 – 431) and fused to Go, the SacI-Nhel fragment from pMD1052 (24) was cloned into SacI-Nhel-cut pMD240, a multicopy plasmid (21). pMD2445 and pMD 284 were transformed sequentially into A232 (21) to yield A4881. A Ste2p plasmid with truncation at residue 369 (Δ369 – 431) was created as a control for this fusion. A double-stranded block of DNA (gBlock gene fragment, Integrated DNA Technologies) containing the sequence from Leu269 to Ala369, followed by three tandemly repeated copies of the influenza HA epitope, was purchased from Integrated DNA Technologies. Yeast homologous transformation was used to incorporate the truncated STE2 gene (KpnI- and NotI-digested gBlock) into KpnI-NotI-digested multicopy plasmid pMD240, creating plasmid pMD2515. pMD 2515 and pMD 284 were transformed sequentially into A232 to yield A4882.

All strains used in this paper were created using the host strains A230 (MATa cryl’ ade2-1 his4-580 lys2Δ trp1Δ tyr1Δ SUP4 – 3Δ leu2Δ ura3Δ bar1-1) or A232, the isogenic ste2Δ strain (21). All strains are FAR1+, allowing normal cell cycle arrest in response to pheromone and are deleted for BAR1, which encodes a protease that degrades α-factor.

To created sst2Δ strains, yeast strains containing either chromosomal STE2+ (A529) or a chromosomal deletion of STE2 (A575) (38) were transformed with pMD524 (pBC14 (35)), as described previously (21), yielding strains A1110 and A1111. These strains were then transformed with an empty vector (pMD228), a CEN plasmid encoding full-length Ste2p (pMD149), or a multicopy plasmid encoding a full-length Ste2p (pMD240) (38). In working with sst2Δ strains, care was taken to pick and subclone colonies of average size as these strains produce colonies of various sizes, presumably due to spontaneous off-target mutations (21).

Ligand Preparation for Assays—All ligand stocks were prepared in a 50% methanol solution in BSA-coated black microcentrifuge tubes (to prevent photobleaching) as described above. The equivalent of 1 × 10⁶ cells was added to ice-cold 20 mM acetate buffer, pH 4.6, sufficient to bring the final volume to 400 μl. 10 μl of the fluorescent ligand [Lys7(NBD),Nle12]α-factor, prepared as described above, was added to the cells and mixed by vortexing. The samples were protected from light and incubated on ice for 30 min, after which binding was measured on FACS Canto II (BD Biosciences). The mean fluorescence emission value for 10,000 cells, excited at 488 nm, was recorded in a channel extending from 515 to 545 nm. Autofluorescence of the yeast cells was determined based on a sample containing 10 μl of 50% methanol without ligand, and this value was subtracted from each sample before analysis. The mean fluorescence value at each ligand concentration was fit to a single site binding equation with a nonspecific component using nonlinear least squares function of SigmaPlot. In every case, separate fitting of three independent isolates was performed, and the mean of the Bmax and Kd values for the three isolates is reported with a standard error of the mean.

Ligand Binding Assays—Saturation binding analysis on all strains was performed using a procedure adapted from Bajaj et al. (25). Yeast cells were grown overnight to an A600 of ~1.0. Fluorescent ligand stocks were prepared in a 50% methanol solution in BSA-coated black microcentrifuge tubes (to prevent photobleaching) as described above. The equivalent of 1 × 10⁶ cells was added to ice-cold 20 mM acetate buffer, pH 4.6, sufficient to bring the final volume to 400 μl. 10 μl of the fluorescent ligand [Lys7(NBD),Nle12]α-factor, prepared as described above, was added to the cells and mixed by vortexing. The samples were protected from light and incubated on ice for 30 min, after which binding was measured on FACS Canto II (BD Biosciences). The mean fluorescence emission value for 10,000 cells, excited at 488 nm, was recorded in a channel extending from 515 to 545 nm. Autofluorescence of the yeast cells was determined based on a sample containing 10 μl of 50% methanol without ligand, and this value was subtracted from each sample before analysis. The mean fluorescence value at each ligand concentration was fit to a single site binding equation with a nonspecific component using nonlinear least squares function of SigmaPlot. In every case, separate fitting of three independent isolates was performed, and the mean of the Bmax and Kd values for the three isolates is reported with a standard error of the mean.

Competition binding assays were performed by adding 10 μl of mixtures of unlabeled antagonist and [Lys7(NBD),Nle12]α-factor to 1.5 × 10⁶ cells, followed by flow cytometric analyses identical to those described above for saturation binding. The mean fluorescence values for each of the concentrations was plotted and fit to a one-site competitive binding curve using the nonlinear least squares function of SigmaPlot. Inhibition constants for each strain were calculated using the Cheng-Prusoff Equation 1 (89) as follows,
where the $K_a$ value is obtained from the saturation binding experiment for the respective strain as described above. The error in $K_i$ (reported as standard error of the mean) was propagated from the corresponding errors in the $IC_{50}$ and $K_a$ values.

The expected dose ratios (DR) based on binding affinities were calculated in Equation 2 (42),

$$\text{DR}_{\text{band}} = 1 + \frac{[\text{antagonist}]}{K_i}$$  \hspace{1cm} (Eq. 2)\\

where $K_i$ is calculated as described above.

The low level of fluorescent signals from strains expressing Ste2p from the chromosomal STE2 locus or from the galactose-repressed STE2 gene makes it difficult to obtain reliable $K_a$ and $IC_{50}$ values for these strains. Thus, for the purposes of dose ratio calculations, $K_i$ values measured for strains expressing STE2 from a CEN plasmid (but lacking a chromosomal copy of STE2) were used to calculate expected dose ratios for these strains.

The expected dose ratios based on measurements of FUS1-lacZ induction (DR$_{\text{ind}}$) were calculated according to Equation 3 (42) and are reported as standard errors of the mean propagated from the numerator and denominator.

$$\text{DR}_{\text{ind}} = \frac{E_{\text{C50}} \text{ in the presence of antagonist}}{E_{\text{C50}} \text{ in the absence of antagonist}}$$  \hspace{1cm} (Eq. 3)

Immunoblotting—Immunoblots for strains overexpressing Sst2p, Gpa1p, or Ste4p were performed as described previously (21). Briefly, $4 \times 10^7$ cells grown to an optical density (600 nm) of $0.75–1.2$ were pelleted by centrifugation and washed with 750 µl of 25 mM Tris, pH 8.0. The cells were resuspended in 200 µl of breaking buffer (40 mM Tris, pH 6.8, 0.1 M EDTA, 5% SDS, 9 M urea, 0.02 mg/ml bromphenol blue, and 2 mM phenylmethylsulfonyl fluoride) and 0.15 g of 0.5-mm zirconia beads and lysed by vortexing for 20 s, followed by 1 min on ice (repeated for 20 cycles). The supernatant was heated at 37°C for 10 min, and 10 µl of the sample were loaded on gradient Tris-HCl 4–15% gel (Bio-Rad). After electrophoresis, the gel was transferred onto a 0.2-µm nitrocellulose membrane overnight. The membranes were blocked with 5% Carnation powdered milk for 2 h, followed by incubation with the primary antibody to TBS + 1% casein + 0.05% Tween 20 for 1 h at room temperature. The primary antibodies were a gift from Dr. Orna Resnekov of the Molecular Sciences Institute and were used at the following dilutions: α-Sst2p (1:3000), α-Gpa1 (1:6000), and α-Ste4 (1:6000). These antibodies have been described previously (90), and their specificities are confirmed by the inclusion of strains deleted for their respective antigens in Figs. 4A and B, and 6A. Following incubation with primary antibodies, the blots were incubated with secondary antibody, HRP-conjugated goat anti-rabbit antibody at 1:10,000 dilution in TBS + 5% milk for 2 h and imaged using Supersignal West Dura chemiluminescent signal (Thermo Scientific). To blot for glucose-6-phosphate dehydrogenase as a loading control, the blots were stripped using Reblot Plus strong solution (EMD Millipore), as per the manufacturer’s protocol. The blot was then re-probed using anti-glucose-6-phosphate dehydrogenase (1:50,000) (Sigma) and HRP-conjugated goat anti-rabbit (1:10,000). Gels were quantified using ImageJ. All protein expression levels were normalized to glucose-6-phosphate dehydrogenase expression levels. The fold over-expression of the desired protein was calculated as the ratio of glucose-6-phosphate dehydrogenase-normalized expression levels between the strain overexpressing the protein and the strain expressing wild-type levels of the protein.

Author Contributions—R. S. and M. E. D. designed the study. R. S. performed most of the experimental studies and wrote much of the manuscript. S. M. C. performed some of the described experiments. F. N. provided critical reagents and participated in writing the manuscript. M. E. D. oversaw the project, analyzed results, and participated in the writing of the manuscript.

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References

1. Klambunde, T., and Hessler, G. (2002) Drug design strategies for targeting G-protein-coupled receptors. Chembiochem 3, 928–944
2. Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006) How many drug targets are there? Nat. Rev. Drug Discov. 5, 993–996
3. Brown, A. J., Dyos, S. L., Whiteway, M. S., White, J. H., Watson, M. A., Marzioch, M., Clare, J. J., Couzens, D. J., Paddon, C., Plumpton, C., Romanos, M. A., and Dowell, S. J. (2000) Functional coupling of mammalian RGS proteins to the yeast mating pathway using novel yeast/mammalian G protein α-subunit chimeras. Yeast 16, 11–22
4. King, K., Dohlman, H. G., Thorner, J., Caron, M. G., and Letkowitz, R. J. (1990) Control of yeast mating signal transduction by a mammalian β2-adrenergic receptor and Gs α subunit. Science 250, 121–123
5. Price, L. A., Kajkowski, E. M., Hadcock, J. R., Ozenberger, B. A., and Pausch, M. H. (1995) Functional coupling of a mammalian somatostatin receptor to the yeast pheromone response pathway. Mol. Cell. Biol. 15, 6188–6195
6. Yin, D., Gavi, S., Shumay, E., Duell, K., Konopka, J. B., Malbon, C. C., and Wang, H.-Y. (2005) Successful expression of a functional yeast G-protein-coupled receptor (Ste2) in mammalian cells. Biochem. Biophys. Res. Commun. 329, 281–287
7. Crowe, M. L., Perry, B. N., and Connerton, I. F. (2000) Golf complements a GPA1 null mutation in Saccharomyces cerevisiae and functionally couples to the STE2 pheromone receptor. J. Recept. Signal Transduct. Res. 20, 61–73
8. Apanovitch, D. M., Slep, K. C., Sigler, P. B., and Dohlman, H. G. (1998) Sst2 is a GTase-activating protein for Gpa1: purification and characterization of a cognate GTS-Gα protein pair in yeast. Biochemistry 37, 4815–4822
9. Dixit, G., Kelley, J. B., Houser, J. R., Elston, T. C., and Dohlman, H. G. (2014) Cellular noise suppression by the regulator of G protein signaling Sst2. Mol. Cell 55, 85–96
10. Hao, N., Yildirim, N., Nagiec, M. J., Parnell, S. C., Errede, B., Dohlman, H. G., and Elston, T. C. (2012) Combined computational and experimental analysis reveals mitogen-activated protein kinase-mediated feedback phosphorylation as a mechanism for signaling specificity. Mol. Biol. Cell 23, 3899–3910
11. Yildirim, N., Hao, N., Dohlman, H. G., and Elston, T. C. (2004) Mathematical modeling of RGS and G-protein regulation in yeast. Methods Enzymol. 389, 383–398
12. Hao, N., Yildirim, N., Wang, Y., Elston, T. C., and Dohlman, H. G. (2003) Regulators of G protein signaling and transient activation of signaling...
Variable Dependence of Signaling Output on Agonist Occupancy

55. Hartwell, L. H. (1980) Mutants of Saccharomyces cerevisiae unresponsive to cell division control by polypeptide mating hormone. J. Cell Biol. 85, 811–822
56. Chasse, S. A., Flanary, P., Parnell, S. C., Hao, N., Cha, J. Y., Siderovski, D. P., and Dohlman, H. G. (2006) Genome-scale analysis reveals new functional regulators of mating pheromone signaling in the yeast Saccharomyces cerevisiae. Eukaryot. Cell 5, 330–346
57. Burchett, S. A., Scott, A., Errede, B., and Dohlman, H. G. (2001) Identification of novel pheromone-response regulators through systematic overexpression of 120 protein kinases in yeast. J. Biol. Chem. 276, 26472–26478
58. Cappell, S. D., Baker, R., Skowyra, D., and Dohlman, H. G. (2010) Systematic analysis of essential genes reveals important regulators of G protein signaling. Mol. Cell 38, 746–757
59. Raser, J. M., and O’Shea, E. K. (2005) Noise in gene expression: origins, consequences, and control. Science 309, 2010–2013
60. Konopka, J. B., Margarit, S. M., and Dube, P. (1996) Mutation of Pro-258 in transmembrane domain 6 constitutively activates the G protein-coupled α-factor receptor. Proc. Natl. Acad. Sci. U.S.A. 93, 6764–6769
61. Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998) Mechanisms governing the activation and trafficking of yeast G protein-coupled receptors. Mol. Biol. Cell 9, 885–899
62. Dolil, M., Giot, L., Davis, C., and Konopka, J. B. (1998) Dominant-negative mutations in the G-protein-coupled α-factor receptor map to the extracellular ends of the transmembrane segments. Mol. Cell. Biol. 18, 5981–5991
63. Büküşoğlu, G., and Jenness, D. D. (1996) Agonist-specific conformational changes in the yeast α-protein pheromone receptor. Mol. Cell. Biol. 16, 4818–4823
64. Abel, M. G., Lee, B. K., Naider, F., and Becker, J. M. (1998) Mutations affecting ligand specificity of the G-protein-coupled receptor for the Saccharomyces cerevisiae tridecapeptide pheromone. Biochem. Biophysics Acta 1448, 12–26
65. Marsh, L. (1992) Substitutions in the hydrophobic core of the α-factor receptor of Saccharomyces cerevisiae permit response to Saccharomyces kluveri α-factor and to antagonist. Mol. Cell. Biol. 12, 3959–3966
66. Lin, J. C., Parrish, W., Ellers, M., Smith, S. O., and Konopka, J. B. (2003) Aromatic residues at the extracellular ends of transmembrane domains 5 and 6 promote ligand activation of the G protein-coupled α-factor receptor. Biochemistry 42, 293–301
67. Ferré, S., Casadó, V., Devi, L. A., Filipzada, M., Jockers, R., Lohse, M. J., Milligan, G., Pin, J. P., and Guitart, X. (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol. Rev. 66, 413–434
68. Whorton, M. R., Bokoch, M. P., Rasmussen, S. G., Huang, B., Zare, R. N., and Dohlman, H. G. (2001) Iden-}
69. Varrault, A., Journot, L., Audigier, Y., and Bockaert, J. (1992) Transfection of human 5-hydroxytryptamine1A receptors in NIH-3T3 fibroblasts: effects of increasing receptor density on the coupling of 5-hydroxytryptamine1A receptors to adenylyl cyclase. Mol. Pharma- mols 41, 999–1007
70. Claesens, S., Sebben, M., Bécamel, C., Eglen, R. M., Clark, R. D., Bockaert, J., and Dumuis, A. (2000) Pharmacological properties of 5-hydroxytryptamine(4) receptor antagonists on constitutively active wild-type and mutated receptors. Mol. Pharmacol. 58, 136–144
71. Lin, J. C., Parrish, W., Ellers, M., Smith, S. O., and Konopka, J. B. (2003) Aromatic residues at the extracellular ends of transmembrane domains 5 and 6 promote ligand activation of the G protein-coupled α-factor receptor. Biochemistry 42, 293–301
72. Ferré, S., Casadó, V., Devi, L. A., Filipzada, M., Jockers, R., Lohse, M. J., Milligan, G., Pin, J. P., and Guitart, X. (2014) G protein-coupled receptor oligomerization revisited: functional and pharmacological perspectives. Pharmacol. Rev. 66, 413–434
73. Whorton, M. R., Bokoch, M. P., Rasmussen, S. G., Huang, B., Zare, R. N., Kobilka, B. K., and Sunahara, R. K. (2007) A monomeric G protein-coupled receptor isolated from a high-density lipoprotein particle efficiently activates its G protein. Proc. Natl. Acad. Sci. U.S.A. 104, 7682–7687
74. Ernst, O. P., Gramse, V., Kolbe, M., Hofmann, K. P., and Heck, M. (2007) Monomeric G protein-coupled receptor rhodopsin in solution activates its G protein transducin at the diffusion limit. Proc. Natl. Acad. Sci. U.S.A. 104, 10859–10864
75. Overton, M. C., Chia and Blumer, K. J. (2003) Oligomerization, biogenesis, and signaling is promoted by a glycoprotein A-like dimerization motif in transmembrane domain 1 of a yeast G protein-coupled receptor. J. Biol. Chem. 278, 49369–49377
76. Overton, M. C., and Blumer, K. J. (2000) G protein-coupled receptors function as oligomers in vitro. Curr. Biol. 10, 341–344
77. Yesilatatay, A., and Jenness, D. D. (2000) Homo-oligomeric complexes of the yeast α-protein pheromone receptor are functional units of endocytosis. Mol. Biol. Cell 11, 2873–2884
78. Kim, H., Lee, B. K., Naider, F., and 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, 10976–10987
79. Marunaka, Y., Nisato, N., and Miyazaki, H. (2005) New concept of spare receptors and effectors. J. Membr. Biol. 203, 31–39
80. Redka, D. S., Heerklotz, H., and Wells, J. W. (2013) Efficacy as an intrinsic property of the M2 muscarinic receptor in its tetrameric state. Biochemistry 52, 7405–7427
81. Han, Y., Moreira, I. S., Urizar, E., Weinstein, H., and Javitch, J. A. (2009) Allosteric communication between protomers of dopaminergic A2A receptors: modulation by G protein. Nat. Chem. Biol. 5, 688–695
82. Zolk, O., Kilter, H., Flesch, M., Mansier, P., Svynghedawu, B., Schnabel, P., and Böhm, M. (1998) Functional coupling of overexpressed β2-adreno- receptors in the myocardium of transgenic mice. Biochem. Biophys. Res. Commun. 248, 801–805
83. Ashkenazi, A., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J., and Ramachandran, J. (1987) An M2 muscarinic receptor subtype coupled to both adenylylcyclase and phosphoinositide turnover. Science 238, 672–675
84. Bouvier, M., Hnatiowich, M., Collins, S., Kobilka, B. K., Deblasi, A., Lefkowitz, R. J., and Caron, M. G. (1988) Expression of a human cDNA encoding the β2-adrenergic receptor in Chinese hamster fibroblasts (CHW): functional expression and regulation of the expressed receptors. Mol. Pharmacol. 33, 133–139
85. Whaley, B. S., Yuan, N., Barber, R., and Clark, R. B. (1995) β-Adrenergic regulation of adenylylcyclase: effect of receptor number. Pharmacol. Commun. 6, 203–210
86. Marunaka, Y., Nisato, N., and Miyazaki, H. (2005) New concept of spare receptors and effectors. J. Membr. Biol. 203, 31–39
87. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27
88. Celis, A., Connelly, S. M., Martin, N. P., and Dumont, M. E. (2004) Intensive mutational analysis of G protein-coupled receptors in yeast. Methods Mol. Biol. 237, 105–120
89. Cheng, Y., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108
90. Pincus, D., Benjamin, K., Burbulis, I., Tsong, A. E., and Resnekov, O. (2010) Reagents for investigating MAPK signalling in model yeast species. Yeast 27, 423–430