Supporting Text

Strain Construction. All gene constructs were transformed into the genome by PCR-based gene integration, and were confirmed by PCR and Western blot (1). CFP was inserted in between residues 10 and 11 of Gpa1p (HIS5-marked vector) and YFP was inserted in between residues 104 and 105 of Ste18p (kanamycin-marked vector) by PCR-based gene integration of RJD415 to create the FRET strain TMY101. The singly labeled strains TMY102 (*CFP-GPA1*) and TMY103 (*STE18-YFP*) contained only one of the two FRET probes. The *sst2*Δ strain TMY91 was constructed by PCR-based gene disruption of the strain RJD360. In the strain TMY92 ($ste2^{300\Delta}$), we inserted a stop codon after amino acid 300 in the *STE2* gene of RJD415 by using PCR-based gene insertion. We crossed TMY91 with TMY101 to place the *sst2*Δ mutation into the FRET background (TMY111). We crossed TMY92 with TMY101 to place the $ste2^{300\Delta}$ mutation into the FRET background (TMY112). One of the sporulation products from the TMY91 × TMY101 cross, TMY113, contained the wild-type genes for both *SST2* and *BAR1*, along with *CFP-GPA1* and *STE18-YFP*. See Table 2 for genotypes of strains.

Ligand-Binding and Receptor Endocytosis Assays. Ligand-binding assays were performed with ^{35}S-labeled α-factor and whole cells in YPAD medium (pH 6.4) treated with 10 mM NaN_3 and 10 mM NaF (2). The α-factor internalization assays were performed essentially as described (3).

Additional Notes on FRET Quantification. The FRET efficiencies in the absence of α-factor, E_0 ($t = 0$), and when G protein activation was at its maximum, E_{max} ($t = 30$s), were determined by using the following formulas: $E_0 = 1 - \frac{F_{DA}^0}{F_D^0}$ and $E_{\max} = 1 - \frac{F_{DA}^{\max}}{F_D^{\max}}$, where F_{DA} is the fluorescence emission of the donor CFP-Gpa1p in the presence of the FRET acceptor Ste18p-YFP, and F_D is the emission of CFP-Gpa1p alone.

For TMY111, $r_0 = 0.63$, and $r_{max} = 0.7$; for TMY112, $r_0 = 0.59$ and $r_{max} = 0.67$. We could not calculate E_0 and E_{max} for these two strains, because we did not have singly labeled CFP-Gpa1p versions of the strains to determine F_D.

Background subtraction (subtracting the intrinsic background fluorescence of yeast cells) was not necessary for the raw 475/530 ratios, r_i, because we were quantitating changes in r_i. Background subtraction was performed on the representative spectra in Fig. 2 and for the determination of E_0 and E_{max}.

The fluorescence emission of CFP-Gpa1p alone and Ste18p-YFP alone did not change significantly during the first 10 min of the pheromone response.

Additional Notes on Parameter Estimation. Siekhaus and Drubin (4) have recently demonstrated that *sst2*Δ cells exhibit spontaneous receptor-independent G protein activation. For the modeling described above, we have assumed that the *sst2*Δ cells in the absence of pheromone are either fully activated in terms of G protein signaling or completely inactive. Only the inactive class of cells responds to α-factor; the active class contributes to a higher pretreatment baseline level of G protein activation.

1. Wach, A. (1996) *Yeast* **12,** 259–265.

2. Blumer, K. J. & Thorner, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4363–4367.

3. Hicke, L. & Riezmann, H. (1996) *Cell* **84,** 277–287.

4. Siekhaus, D. E. & Drubin, D. G. (2003) *Nat. Cell Biol.* **5,** 231–235.