Subunits of a yeast oligomeric G protein-coupled receptor are activated independently by agonist but function in concert to activate G protein heterotrimers

Sharon L. Chinault, Mark C. Overton, and Kendall J. Blumer*

Department of Cell Biology and Physiology
Washington University School of Medicine
660 S. Euclid Ave.
St. Louis, MO 63110

Contact information for corresponding author*
Ph. 314-362-1668
Fax 314-362-7463
Email kblumer@cellbio.wustl.edu
SUMMARY

G protein-coupled receptors (GPCRs) form dimeric or oligomeric complexes in vivo. However, the function of oligomerization in receptor-mediated G protein activation is unclear.

Previous studies of the yeast α-factor receptor (STE2 gene product) have indicated that oligomerization promotes signaling. Here we have addressed the mechanism by which oligomerization facilitates G protein signaling by examining the ability of ligand binding- and G protein coupling-defective α-factor receptors to form complexes in vivo and to correct their signaling defects when co-expressed (trans complementation). Newly and previously identified receptor mutants indicated that ligand binding involves the exofacial end of TM4, whereas G protein coupling involves ic1, ic3, the C-terminal tail and the intracellular ends of TM2 and 3.

Mutant receptors bearing substitutions in these domains formed homooligomeric or heterooligomeric complexes in vivo as indicated by results of fluorescence resonance energy transfer (FRET) experiments. Co-expression of ligand binding- and G protein coupling-defective mutant receptors did not significantly improve signaling. In contrast, co-expression of ic1 and ic3 mutations in trans but not in cis significantly increased signaling efficiency.

Therefore, we suggest that subunits of the α-factor receptor: 1) are activated independently rather than cooperatively by agonist; and 2) function in a concerted fashion to promote G protein activation, possibly by contacting different subunits or regions of the G protein heterotrimer.
INTRODUCTION

As the largest class of receptors in humans, G protein-coupled receptors (GPCRs) elicit cellular responses to neurotransmitters, hormones, light, and other stimuli. Many GPCRs have been shown recently to exist as dimers/oligomers in vivo (reviewed in refs. (1-3). In addition to promoting exit of GPCRs from the endoplasmic reticulum (4-9), oligomerization appears to be important for GPCR signaling. For example, transmembrane domain peptides that inhibit oligomerization of β2-adrenergic receptors can attenuate signal transduction in vitro (10), and oligomerization-defective mutants of a yeast GPCR can bind agonist normally but are signaling impaired (11). Strikingly, heterodimerization between the R1 and R2 subunits of the GABA(B) receptor is essential for signal transduction because the R1 subunit can bind agonist but cannot activate G proteins, whereas the R2 subunit cannot bind agonist but can activate G proteins (5, 12-15).

Studies of chimeric and point mutant forms of various GPCRs have suggested at least three mechanisms by which receptor oligomerization could promote G protein activation (Figure 1A). In the one model, agonist binding to one receptor subunit induces a conformational change that is transmitted allosterically to another receptor subunit, which in turn undergoes a conformational change resulting in G protein activation. This mechanism has been suggested by studies of the GABA(B) receptor heterodimers (16-20), by cooperative agonist binding to
receptors such as muscarinic acetylcholine receptors (21, 22), and by studies indicating that heterodimerization can alter the signaling, trafficking or desensitization of opioid, β-adrenergic, bradykinin or angiotensin II receptors (23-27). In a second model suggested by studies of GABA(B) receptors (16-20), agonist binding to R1 subunit induces movement that relieves its inhibitory action on the R2 subunit, which is freed for G protein activation. In a third model, suggested by studies of leutinizing hormone receptors (28, 29), \textit{trans} activation of one receptor by another can involve exchange of ligand-binding domains.

In contrast, other lines of evidence suggest that subunits of GPCR oligomers may function independently in the process of G protein activation. For example, only one molecule of the photoreceptor rhodopsin, which forms arrays of homodimers in disk membranes (30, 31), is activated by a single photon (reviewed in ref. (32)). Likewise, agonist binding to many GPCRs is not cooperative. Furthermore, GPCRs defective in agonist binding or G protein coupling generally are not dominant negative; those that are dominant negative usually interfere with wild type receptor function by sequestering G proteins or retaining wild type receptors in nonproductive complexes in the endoplasmic reticulum (4, 6, 33). There are exceptions, however. A DRY motif mutant of the CCR2 receptor is dominant negative, apparently because it forms non-productive complexes with wild type receptors on the cell surface (34).
Because the functions of GPCR oligomerization in G protein activation remain unclear for most receptors, we have sought to provide new insight by analyzing the yeast α-factor receptor (STE2 gene product). This receptor forms constitutive (i.e. agonist-independent) oligomeric complexes in vivo and oligomerization-defective mutants bind agonist normally but are signaling defective (35-37), suggesting that oligomerization promotes G protein activation. Furthermore, the α-factor receptor is a useful model because mechanisms of receptor-G protein signaling are conserved from yeast to humans (reviewed in ref. (38)). Here we have identified new mutants defective in G protein coupling, and used them and previously described ligand-binding and G protein-coupling mutants to determine whether receptors with different classes of signaling defects self-associate or form heterooligomeric complexes with one another. We also used this collection of mutants to determine whether receptor function is reconstituted when different classes of mutant receptors are co-expressed, a process termed trans complementation. The results suggest that each α-factor receptor subunit is activated independently by agonist and that receptor subunits function in cooperation with one another to activate G protein heterotrimerics.
EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids - The *S. cerevisiae* strains used in these studies were KBY58 (*MATα leu2-3,112 ura3-52 his3-Δ1 trp1 ste2::leu2 sst1-Δ5*) and JE114-8A (*MATα leu2,3-112 ura3-52 his3Δ1 trp1 ste2::LEU2 sst2-1*). Cells were grown in synthetic medium containing adenine and amino acid supplements, but lacking tryptophan, histidine, or uracil to maintain plasmids. All mutations were made in the single copy plasmid pRS314Ste2-3myc (39), which uses native *STE2* promoter and terminator sequences to drive expression of full-length or C-terminally truncated (at codon 303) α-factor receptors tagged at their C-termini with three copies of the c-myc epitope.

Oligonucleotide-Directed Mutagenesis - All mutations were introduced into pRS314Ste2-3myc using the QuikChange™ site-directed mutagenesis method (Stratagene). An *HpaI-AatII* fragment bearing a mutation of interest was used to replace the equivalent fragment of pRS314Ste2Δtail-3myc to obtain versions of the mutant receptors truncated at position 303. All mutations were subsequently subcloned into pRS313 or pRS426 as *XhoI-SpeI* fragments. To construct plasmids expressing tailless wild type or mutant receptors tagged with CFP or YFP, we either subcloned the *HpaI-AatII* fragment bearing a mutation of interest into pRS423Ste2Δtail-YFP and pRS424Ste2Δtail-CFP, or used site-directed mutagenesis to introduce desired mutations.
into pRS423Ste2Δtail-YFP and pRS424Ste2Δtail-CFP. All mutations were verified by DNA sequencing.

Assays of Agonist Response – Assays of α-factor-induced growth arrest (halo assays) were used to assess the function of wild type or mutant receptors expressed at native levels from single copy plasmids or over-expressed on high copy (2μ) plasmids in cells bearing a deletion of the chromosomal gene encoding the α-factor receptor (STE2). Results of dose-response experiments were used to quantify the relative sensitivities of cells expressing mutant receptors to agonist stimulation. Shorter-term assays (2h) of agonist response (transcriptional induction of a FUS1-lacZ reporter in plasmid pSL307) were performed as described (40). Data were analyzed by nonlinear regression using GraphPad Prism v.3.0a.

Preparation of 35S-Labeled α-Factor and Agonist-Binding Assays - Methods used to purify [35S]α-factor from metabolically labeled yeast cells and to perform ligand-binding assays with inviable, intact cells are identical to those described previously (39, 41). Agonist binding data were analyzed according to the method of Scatchard and fitted by nonlinear least-squares regression. Nonspecific binding was determined in the presence of a 200-fold excess of unlabeled α-factor.
Fluorescence Resonance Energy Transfer (FRET) - Scanning fluorometry of intact viable yeast cells co-expressing tailless forms of CFP- and YFP-tagged wild type or mutant α-factor receptors was used to detect FRET between oligomerized receptors in vivo, as previously described in detail (29, 31, 37). Briefly, cells were suspended in 25mM Tris-HCl pH 7.5 and irradiated in 3ml glass cuvettes. Cells were irradiated at 425nm to excite CFP and fluorescence emission was recorded from 450-610nm, or irradiated at 510nm to excite YFP and fluorescence emission was recorded from 520-610nm. The FRET component of the emission spectrum obtained from cells co-expressing CFP- and YFP-tagged receptors was obtained by subtracting components due to CFP emission and YFP emission produced by direct excitation at 425nm. The apparent efficiency of FRET was calculated by dividing the peak FRET value by the peak value obtained upon direct excitation of YFP (510nm).

Localization of GFP-Tagged Receptors - Fluorescence images of cells expressing wild type and various receptor mutants tagged with GFP were captured by using a DAGE cooled CCD camera mounted on an Olympus BH-2 or IX81 microscope equipped with a PlanApo100UV 100X objective, as described previously (38).
RESULTS

Use of Trans-Complementation to Assess Functional Interaction Between Subunits of α-Factor Receptor Oligomers—Strong evidence indicating that signaling or functional coupling occurs between subunits of a GPCR complex has come from studies of GABA(B) receptor heterodimers. Whereas each subunit of this receptor has an intrinsic signaling defect (G protein coupling defect for the R1 subunit and an agonist binding defect for the R2 subunit), these signaling defects are complemented to produce a functional receptor when R1 and R2 are co-expressed in trans. We therefore chose to use an analogous approach to determine whether co-expression of different classes of mutant α-factor receptors could restore receptor function by interacting with one another (trans complementation). Accordingly, we needed a collection of mutant receptors defective in various aspects of receptor function. Because relatively few signaling-defective α-factor receptor mutants have been identified and characterized previously, we initially generated a collection of mutant α-factor receptors likely to be defective in G protein coupling. These mutants and previously described agonist-binding and G protein-coupling mutants could then be used to determine whether co-expressed mutant receptors could associate with one another in fluorescence resonance energy transfer (FRET) experiments, and rescue receptor function by a trans complementation mechanism.
Identification of New α-Factor Receptor Mutants Apparently Defective in G Protein

Coupling- Because the third intracellular loop (ic3) of the α-factor receptor is known to promote G protein coupling (4244), we sought to identify new G protein coupling mutants by investigating the first or second intracellular loops (ic1 or ic2) or adjacent portions of their appended transmembrane domains (Figure 1B). Extensive mutagenesis of these domains in the context of full-length receptors failed to identify mutants with a significant signaling defect. For example, in three mutants we replaced triplets of amino acids with alanine residues in the cytoplasmic-proximal portions of TM2 (80-2A) or TM3 (151-3A and 153-5A), and in a fourth mutant (ic1A) we replaced most of ic1 (residues 72-78) with alanine residues (Figure 1B). None of these substitutions had a significant effect (>2- 3-fold; Figure 2) on signaling efficiency, as indicated by formation of clear zones of growth inhibition of similar size to that obtained with wild type receptors when cells were challenged with the same dose of α-factor (15 nmol).

Therefore, we hypothesized that these or other domains may normally function in cooperation with one another to promote G protein activation.

To test this hypothesis, we repeated the mutagenesis of ic1, ic2 and adjacent regions of their appended TM domains in the context of receptors lacking their cytoplasmic C-terminal domain (truncated at position 303 following TM7; Figure 1B). This truncation mutant was used because the C-terminal domain promotes formation of preactivation complexes with G protein
heterotrimers (45), yet is dispensable for G protein activation and signaling (46). Truncated receptors therefore form weakened but functional preactivation complexes, which may allow other receptor domains involved in G protein coupling and signaling to be identified by mutagenesis.

Using this approach we identified four receptor mutants that potentially had G protein coupling defects (Figure 1B and Figure 2). In three mutants, triplets of amino acids were replaced with alanine residues in the cytoplasmic-proximal portions of TM2 (80-2A) or TM3 (151-3A and 153-5A). Whereas this region of TM2 had not been identified previously as being important for receptor signaling, the end of TM3 had been implicated in a recent study (47). A fourth signaling-deficient mutant (ic1A) involved replacing most of ic1 (residues 72-78) with alanine residues, a domain that had not been implicated previously as being involved in α-factor receptor signaling.

The phenotypes of cells expressing tailless forms of these mutant receptors from single-copy plasmids and their normal promoters were assessed initially by performing assays of agonist-induced growth arrest (Figure 2). The results indicated that expression of tailless mutant receptors significantly impaired signaling, as indicated by the formation of turbid or smaller zones of growth inhibition in response to a fixed dose of agonist (15 nmol). In contrast,
mutations affecting ic2 had no effect, including one in which all of ic2 was replaced by alanine residues (residues 157-162; data not shown).

To further analyze the effects of these newly identified mutations on receptor function, we characterized them with respect to the magnitudes of their signaling defects, cell-surface expression and agonist binding affinities. Signaling defects were assessed initially by performing experiments using agonist-induced growth arrest as an assay. These experiments used cells lacking the yeast RGS protein, Sst2, which promotes recovery from agonist-induced growth arrest by stimulating the ability of Gα subunits to hydrolyze GTP (48). In this genetic background, zones of agonist-induced growth inhibition would be clear due to impaired recovery caused by lack of Sst2 function. Indeed, expression of tailless forms of mutant receptors in the sst2 mutant background resulted in the appearance of smaller, clear zones of growth inhibition in response to a fixed dose of agonist (15nmol; Figure 2), indicating impaired cell responsiveness. By varying the dose of agonist, we generated dose-response curves for agonist-induced growth arrest of cells expressing tailless forms of wild type and mutant receptors (Figure 3A). These curves were used to calculate the dose of agonist needed to produce a zone of growth inhibition 20mm in diameter. These values were normalized to that calculated for cells expressing wild type tailless receptors, yielding values of relative sensitivity. This analysis indicated that cells expressing tailless forms of mutant receptors were less sensitive to agonist, as follows: ic1A,
0.05-fold; 80-2A, 0.11-fold; 151-3A, 0.13-fold; 153-5A, 0.06-fold.

Because assays of agonist-induced growth arrest are long-term (2d) indicators of signaling function, we also performed dose-response experiments using an agonist-inducible reporter gene (FUS1-lacZ) as a shorter-term (2h) read out of signaling activity (Figure 3B). The results indicated that the apparent EC\textsubscript{50} values for cells expressing tailless forms of wild type or mutant receptors were: WT=3nM; ic1A=18nM; 80-2A=12nM; 151-3A=17nM; 153-5A=41nM. Therefore, cells expressing mutant receptors were 0.25- to 0.07-fold as sensitive to agonist stimulation as cells expressing wild type tailless receptors, similar to the results of growth arrest assays.

To determine whether mutant receptors had defects in agonist-binding affinity and/or cell surface expression, we performed equilibrium binding assays with \textsuperscript{35}S-labeled \(\alpha\)-factor and intact, inviable cells expressing tailless versions of wild type and mutant receptors. As a complementary approach we also analyzed the subcellular localization of GFP-tagged tailless mutant receptors. The results of agonist binding assays indicated that each receptor mutant displayed a single class of binding site with an affinity similar to that of wild type receptors (Table 1). These results also indicated that the cell surface expression levels of two receptor mutants (151-3A and 153-5A) were nearly normal whereas those of two other mutants (ic1A and
80-2A) were 10-fold lower than wild type (Table 1). These results were consistent with those obtained by analyzing the subcellular localization of GFP-tagged mutant receptors by fluorescence microscopy (Figure 4). However, defects in cell surface expression were unlikely to account for the signaling deficits of the mutant receptors, because receptor overexpression from high copy plasmids did not improve signaling efficiency (Figure 2). Furthermore, previous studies have shown that a 20-fold reduction in expression of wild type receptors does not affect the efficiency of signaling (49). Therefore, the results suggested that mutations affecting ic1 and the cytoplasmic-proximal regions of TM2 and TM3 are likely to impair signaling by reducing the efficiency with which receptors couple with or activate G proteins. Accordingly, these mutants and previously described receptor mutants impaired with respect to G protein coupling (L236R in ic3 (42); Figure 1B) and ligand binding (S184R in the exofacial portion of TM4 (36); Figure 1B) could be used for FRET and trans-complementation experiments.

**Mutant Receptors Efficiently Form Homooligomeric and Heterooligomeric Complexes**

To be useful in trans complementation experiments, mutant receptors must retain the ability to oligomerize. To address this requirement, we performed FRET experiments using living cells to determine whether mutant receptors could self-associate, and whether one type of mutant receptor could associate with another. These experiments used tailless receptors tagged with CFP or YFP as described previously (35). Full-length CFP- and YFP-tagged receptors could not
be used for FRET experiments because this increases the distance between and/or orientational flexibility of the fluorophores, resulting in the loss of a signal (35).

As shown in Figure 5, each of the newly identified mutant receptors self-associated efficiently. Moreover, previously described mutant receptors defective in ligand binding (S184R) or G protein coupling (L236R) self-associated efficiently (Figure 5). Quantification of the FRET data (Table 2) indicated that the 80-2A, 151-3A, 153-5A, S184R and L236R mutants self-associated with efficiencies similar to that of wild type receptors. The ic1A mutant appeared to self-associate somewhat more efficiently than wild type receptors (Table 2). However, we do not believe that this apparent increase is significant because the ic1A mutant receptors tagged with CFP and YFP were expressed at lower levels, which increased the standard deviation of the data (Table 2). Nevertheless, the results support the conclusion that all of the mutant receptors could self-associate with apparent efficiencies similar to that of wild type receptors, and that oligomerization does not require normal G protein-coupling or ligand-binding activity.

To determine whether mutant receptors could form heteromeric complexes with one another, we performed FRET experiments with cells co-expressing representative pairs of mutant receptors of different classes (Figure 6 and Table 2). Again, the results indicated that each of the combinations tested could form complexes with apparent efficiencies similar to that observed
with wild type receptors. Therefore, because the receptor mutants efficiently formed homo- or heterooligomeric complexes, they could be used for trans complementation experiments.

Co-Expression of Certain Pairs of Mutant Receptors Partially Reconstitutes Receptor Function—To address whether the signal of agonist binding can be transferred from one receptor to another, or whether receptor subunits must cooperate with one another to activate G protein heterotrimers, we co-expressed all possible pair-wise combinations of tailless mutant receptors. These experiments used cells lacking Sst2 because this strain yielded zones of agonist-induced growth inhibition that were clear and easily measured, as described previously. As shown in Figure 7, co-expression of each of the pairs of mutant receptors resulted in zones of growth inhibition that were significantly smaller than obtained with cells expressing wild type tailless receptors, indicating that complete restoration of receptor function by trans complementation between mutant receptors did not occur.

To determine whether co-expression of pairs of mutant receptors could partially improve signaling efficiency, we performed dose-response growth arrest experiments like those shown in Figure 3A with cells expressing each mutant receptor individually as well as with cells that co-expressed all pair-wise combinations of mutant receptors. The sensitivity of cells expressing a single type of mutant receptor or cells co-expressing a pair of mutant receptors was normalized
to the sensitivity of cells expressing wild type tailless receptors (Table 3, column 2), as described previously for data shown in Figure 3A. We then normalized the sensitivity of cells co-expressing a given pair of receptors to that obtained with cells expressing only the receptor of the pair which had the greater residual function. For example, the sensitivity of cells co-expressing the S184R and the ic1A mutant receptors (0.13; Table 3) was divided by the sensitivity of cells expressing the ic1A mutant receptor (0.05) rather than by the sensitivity of cells expressing the less functional S184R receptor (0.005; Table 3). The resultant ratio of 2.6 (i.e. 0.13/0.05) indicated that cells co-expressing these two mutant receptors responded to agonist somewhat more efficiently than cells expressing either mutant receptor alone. Thus, this was interpreted as evidence of weak trans complementation.

Using this approach, we obtained two types of results. In most cases, there was evidence of weak or insignificant trans complementation when different types of mutant receptors were co-expressed (ratio of <3 in column 3 of Table 3). This type of result was obtained when cells co-expressed the ligand-binding defective receptor (S184R) with any of the G protein-coupling mutants (Table 3). This result suggested that the signal of agonist binding was not significantly transferred from the G protein coupling-defective mutant to the agonist binding-defective mutant, or that if such intersubunit communication occurred it was insufficient to significantly
improve the efficiency of G protein activation. Presently we favor the former hypothesis because agonist binding to the receptor is not cooperative (41).

In contrast, there were two instances in which stronger evidence of trans complementation was observed (ratio of >3 in column 3 of Table 3). This occurred when cells co-expressed an ic3 mutant (L236R) and the ic1A mutant (ratio = 6.0), or an ic3 mutant (L236R) and the 80-2A (affecting the endofacial region of TM2) mutant (ratio = 3.0). These results suggesting somewhat stronger trans complementation appeared to be specific because co-expression of pairs of receptors with substitutions affecting other intracellular domains produced smaller or insignificant effects (ratios <3.0; column 3, Table 3). Therefore, evidence of trans complementation between the ic1 and ic3 mutant was consistent with a model in which signaling efficiency was partially improved because the G protein-coupling site affected in one mutant receptor is provided by the other. This would be consistent with a mechanism in which receptor subunits normally cooperate with one another to activate G protein heterotrimers.

To address whether the lack of the receptor cytoplasmic tail in the preceding experiments might have affected the results, we determined whether co-expression of full-length forms of mutant receptors could rescue function. This could be done only with an ic3 mutant (L236R) and a ligand binding mutant (S184R), because as discussed previously the other mutations did
not have a phenotype in the context of full-length receptors. Consistent with the results using tailless forms of these mutants receptors in trans complementation assays, there was no evidence of significant functional rescue when full-length forms of the ic3 and ligand binding mutant receptors were co-expressed (ratio in column 3 of Table 3 was 1.1).

To confirm the results obtained from growth arrest assays, we analyzed selected pairs of co-expressed mutant tailless receptors in shorter-term assays of pheromone-induced gene expression. Co-expressed pairs of mutant receptors included those that exhibited evidence of trans complementation activity in growth arrest assays (ic1A + L236R; 80-2A + L236R), and two that apparently did not (ic1A + 80-2A; S184R + L236R). Controls included cells expressing only wild type tailless receptors or a single type of mutant tailless receptor. Each receptor or combination of receptors was analyzed by performing agonist dose-response experiments measuring Fus1-lacZ expression in an sst2-1 mutant (Figure 8 and Table 4). As indicated by EC50 values (Table 4), receptor function similar to that obtained with wild type tailless receptors expressed alone was observed when the following pairs of mutant tailless receptors were co-expressed: ic1A + 80-2A in TM2 (Figure 8A); ic1A + L236R in ic3 (Figure 8B); and 80-2A in TM2 + L236R in ic3 (Figure 8C). In contrast, receptor function was reconstituted poorly when cells co-expressed tailless receptors defective in G protein coupling (L236R in ic3) and agonist binding (S184R in TM4).
Results of FUS1-lacZ expression experiments were analyzed further to determine apparent efficiencies of trans complementation. Data were analyzed by a procedure analogous to that employed previously with growth arrest data. First, we used EC\textsubscript{50} values (Table 4, column 1) to determine the sensitivity of cells co-expressing mutant tailless receptors relative to cells expressing only wild type tailless receptors (Table 4, column 2). We then determined the ratio of the sensitivity of cells co-expressing a given pair of tailless mutant receptors to the sensitivity of cells expressing only the more functional of the two mutant receptors (Table 4, column 3). These ratios indicated that three pairs of mutant receptors exhibited significant trans complementation activity (ratio > 3): ic1A + 80-2A in TM2 (ratio = 5.0); ic1A + L236R in ic3 (ratio = 8.7); and 80-2A in TM2 + L236R in ic3 (ratio = 8.6). One pair exhibited insignificant trans complementation activity: L236R + S184R (ratio = 2.0). Therefore, with one exception the results of gene expression assays were consistent with those from growth arrest assays. The exception was ic1A + 80-2A, in which trans complementation was detected in gene expression assays (Table 4) but not in growth arrest assays (Table 3). The reason for this difference was not clear because as otherwise documented herein the results of growth arrest and gene expression assays were concordant. Therefore, whereas we could not be certain whether the ic1A and 80-2A mutant receptors complemented in trans, there was consistent evidence indicating that trans
complementation occurred between the ic1A and L236R mutants, and the 80-2A and L236R mutants, but not between the L236R and S184R mutants.

**Double Mutations Affecting a Single Receptor Cause Additive Signaling Defects**

As a further control for *trans* complementation experiments, we determined the consequences of combining various pairs of mutations in *cis* within the context of a gene encoding a single receptor. The rationale for doing so was that if two mutations complemented one another in *trans* but not in *cis*, then we could conclude that *trans* complementation occurs because one mutant receptor subunit in the oligomer provides a function impaired in the other mutant receptor, rather than by a domain swapping mechanism.

Accordingly, for these experiments we generated and expressed all possible intragenic double mutant receptors individually from single copy plasmids and their normal promoter in *sst2* mutant cells lacking the chromosomal gene encoding α-factor receptors. As indicated by the results of agonist-induced growth arrest assays, combining pairs of mutations in *cis* always caused greater signaling deficits than were caused by either of the two parental single mutations expressed individually (Figure 9). Therefore, there was no evidence of *cis* complementation, even with the intragenic double mutant (L236R,ic1A) corresponding to the pair of mutations that complemented most strongly when expressed in *trans*. Furthermore, results of agonist binding
assays indicated that the intragenic L236R,ic1A double mutant expressed a receptor that had normal agonist binding affinity and was expressed at the cell surface at levels similar to the corresponding single mutants (Table 1). Therefore, the further impairment of signaling observed with this double mutant relative to either single mutant was likely to be caused by the additive effects of the two mutations on G protein coupling. These results further reinforced the conclusion that G protein activation involves functional cooperation between receptor subunits in an oligomeric complex. In conclusion, the results most strongly support a model in which each α-factor receptor subunit is activated independently by agonist and that activated receptor subunits cooperate to activate G proteins.
DISCUSSION

Here we have provided new insight into the mechanisms by which oligomerization promotes G protein activation by the yeast α-factor receptor. We found that ligand-binding and G protein-coupling receptor mutants can interact efficiently in in vivo FRET experiments, yet their co-expression in trans does not significantly improve receptor function. Therefore, we suggest that each receptor subunit is activated independently by agonist rather than primarily by an allosteric or domain exchange mechanism. Furthermore, because we found that substitutions affecting ic1 and ic3 partially reconstitute receptor function when co-expressed in trans but not in cis, we suggest that receptor subunits function in cooperation with one another to activate G protein heterotrimers.

This model is consistent with previous findings. First, agonist binding to α-factor receptors is not cooperative (41). Second, the model is not invalidated by previous studies showing that co-expression of wild type and mutant α-factor receptors can modify signaling by a G protein sequestration mechanism. For example, the ability of wild type α-factor receptors to interfere with agonist-independent signaling by constitutively active receptors (39) requires the wild type receptor C-terminal cytoplasmic domain (45), which promotes the formation of preactivation complexes between receptors and G proteins. Similar evidence indicates that G
protein sequestration is the mechanism by which dominant-negative α-factor receptor mutants exert their inhibitory effects ((45); M. Overton, unpublished data).

If each α-factor receptor subunit is activated independently by agonist binding, why is oligomerization required for efficient signaling, as indicated by the signaling impairment of oligomerization-defective receptor mutants (11)? On the one hand, oligomerization could stabilize receptors in their agonist-induced active conformation. Alternatively, oligomerization may be required to assemble a functional G protein interaction surface. The latter model is consistent with the observation that the longest dimension of the cytoplasmic face of rhodopsin is barely great enough to span the receptor interaction surface of its G protein, transducin (50). This model is also supported by our observation that G protein coupling-defective α-factor receptor mutants in ic1 and ic3 can partially complement one another in trans. However, because trans complementation between these mutants is incomplete, the ic1 and/or ic3A mutant receptors may also be partially defective in the ability to undergo agonist-induced conformational changes. Such defects might not be rescued fully by co-expression of the ic1 and ic3 mutants because allosteric coupling between receptor subunits, if it occurs at all, is inefficient.

Conclusions suggested by studies of the α-factor receptor are consistent with
investigations of other receptor systems. For example, co-expression of different point mutant forms of the angiotensin II (AT1) receptor can restore ligand-binding activity but not signaling (51), indicating that complete trans complementation does not occur. Whether restoration of ligand binding occurs by transmembrane domain swapping between receptors (52), or by one mutant receptor providing the “address” site for agonist binding and the other the “message” site (53) is not clear.

In contrast, evidence suggesting allosteric signal transfer between mammalian GPCRs has been inferred from other investigations. For example, agonist binding to GPCRs such as cardiac muscarinic acetylcholine receptors is cooperative (21, 22). Furthermore, a recent investigation using α1b-adrenergic or histamine H1 receptors fused at their C-termini with Gα11 has shown that G11 activation occurs upon co-expression of one fusion consisting of wild type G11 and a receptor defective in G protein activation with a second fusion protein consisting of a wild type receptor and mutant G11 that cannot release GDP (54). This study also showed that a fusion consisting of TM1 of the α1b-adrenergic receptor and G11 could associate with wild type α1b-adrenergic receptors but that agonist stimulation did not activate the TM1-G11 fusion. Together these results are consistent with a trans activation mechanism. Therefore, trans activation may be an important signaling mechanism used by some GPCRs. However, results obtained from studies of receptor-G11 fusions can be interpreted differently according to a
model in which the two complementary G11-receptor fusions interact, allowing the functional form of G11 linked to the defective receptor to be exchanged to the functional receptor.

According to this interpretation, the TM1-G11 fusion and α1b-adrenergic receptor associate but G11 exchange to the receptor does not occur because the linker employed (two amino acids) between the end of TM1 and G11 is too short or presents G11 in an unfavorable orientation.

Although allosteric activation between subunits of GPCRs seems unlikely to be a universally used signaling mechanism, there is growing evidence that dimerization/oligomerization is crucial for receptor-mediated G protein activation. In striking support of this concept, a dimer of the purified leukotriene B4 receptor has been shown to interact with a single G protein heterotrimer, forming a pentameric assembly in which agonist-stimulated G protein activation can occur (55). In contrast, the monomeric form of the purified leukotriene B4 receptor monomer could not associate with or activate a G protein heterotrimer (55). Biochemical systems like this will provide the means to determine the precise mechanisms by which GPCR dimers/oligomers are activated by agonists which in turn activate G proteins.

In conclusion, much remains to be understood about the functions of GPCR oligomerization in signaling. How a GPCR dimer/oligomer activates a G protein heterotrimer remains unknown. Moreover, the existence of oligomeric arrays of GPCRs could serve various
functions including amplification, spatial restriction, or differential coupling with various
effectors or regulatory molecules. Likewise, the existence of heterooligomeric assemblies of
GPCRs may provide a mechanism for signaling pathway cross-talk or increasing the functional
diversity of GPCRs.
Acknowledgements—This study was supported by NIH grant GM44592 (K.J.B) and an American Heart Association Predoctoral Fellowship (S.L.C.). We thank T. Baranski for comments on the manuscript.
The abbreviations used are: FRET, fluorescence resonance energy transfer; G protein, guanine nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; RGS protein, regulator of G protein signaling protein, transmembrane domain.
REFERENCES

1. Gomes, I., Jordan, B. A., Gupta, A., Rios, C., Trapaidze, N., and Devi, L. A. (2001) *J Mol Med* **79**, 226-42.

2. Angers, S., Salahpour, A., and Bouvier, M. (2002) *Annu Rev Pharmacol Toxicol* **42**, 409-35.

3. George, S. R., O'Dowd, B. F., and Lee, S. P. (2002) *Nat Rev Drug Discov* **1**, 808-20.

4. Benkirane, M., Jin, D. Y., Chun, R. F., Koup, R. A., and Jeang, K. T. (1997) *J Biol Chem* **272**, 30603-6.

5. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H. (1998) *Nature* **396**, 679-82.

6. Zhu, X., and Wess, J. (1998) *Biochemistry* **37**, 15773-84.

7. Lee, S. P., O'Dowd, B. F., Ng, G. Y., Varghese, G., Akil, H., Mansour, A., Nguyen, T., and George, S. R. (2000) *Mol Pharmacol* **58**, 120-8.

8. Karpa, K. D., Lin, R., Kabbani, N., and Levenson, R. (2000) *Mol Pharmacol* **58**, 677-83.

9. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) *Neuron* **27**, 97-106.

10. Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) *J Biol Chem* **271**, 16384-92.

11. Overton, M. C., Chinault, S. L., and Blumer, K. J. (2003) *J Biol Chem* **23**, 23.

12. Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., and Bettler, B. (1998) *Nature* **396**, 683-7.

13. Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., Yao, W. J., Johnson, M., Gunwaldsen, C., Huang, L. Y., Tang, C., Shen, Q., Salon, J. A., Morse, K., Laz, T., Smith, K. E., Nagarathnam, D., Noble, S. A., Branchek, T. A., and Gerald, C. (1998) *Nature* **396**, 674-9.
14. Ng, G. Y., Clark, J., Coulombe, N., Ethier, N., Hebert, T. E., Sullivan, R., Kargman, S., Chateauneuf, A., Tsukamoto, N., McDonald, T., Whiting, P., Mezey, E., Johnson, M. P., Liu, Q., Kolakowski, L. F., Jr., Evans, J. F., Bonner, T. I., and O'Neill, G. P. (1999) *J Biol Chem* **274**, 7607-10

15. Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A., and Kornau, H. C. (1999) *Science* **283**, 74-7

16. Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., and Pin, J. P. (2001) *Embo J* **20**, 2152-9.

17. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2001) *Proc Natl Acad Sci U S A* **98**, 14643-8.

18. Robbins, M. J., Calver, A. R., Filippov, A. K., Hirst, W. D., Russell, R. B., Wood, M. D., Nasir, S., Couve, A., Brown, D. A., Moss, S. J., and Pangalos, M. N. (2001) *J Neurosci* **21**, 8043-52.

19. Havlickova, M., Prezeau, L., Duthey, B., Bettler, B., Pin, J. P., and Blahos, J. (2002) *Mol Pharmacol* **62**, 343-50.

20. Kniazeff, J., Galvez, T., Labesse, G., and Pin, J. P. (2002) *J Neurosci* **22**, 7352-61.

21. Mattera, R., Pitts, B. J., Entman, M. L., and Birnbaumer, L. (1985) *J Biol Chem* **260**, 7410-21.

22. Wreggett, K. A., and Wells, J. W. (1995) *J Biol Chem* **270**, 22488-99

23. AbdAlla, S., Lother, H., and Quitterer, U. (2000) *Nature* **407**, 94-8.

24. Jordan, B. A., Trapaidze, N., Gomes, I., Nivarthi, R., and Devi, L. A. (2001) *Proc Natl Acad Sci U S A* **98**, 343-8.

25. Mellado, M., Rodriguez-Frade, J. M., Vila-Coro, A. J., Fernandez, S., Martin de Ana, A., Jones, D. R., Toran, J. L., and Martinez, A. C. (2001) *Embo J* **20**, 2497-507.
26. Pfeiffer, M., Koch, T., Schroder, H., Klutzny, M., Kirscht, S., Kreienkamp, H. J., Hollt, V., and Schulz, S. (2001) *J Biol Chem* **276**, 14027-36.

27. Pfeiffer, M., Koch, T., Schroder, H., Laugsch, M., Hollt, V., and Schulz, S. (2002) *J Biol Chem* **277**, 19762-72.

28. Osuga, Y., Hayashi, M., Kudo, M., Conti, M., Kobilka, B., and Hsueh, A. J. (1997) *J Biol Chem* **272**, 25006-12.

29. Ji, I., Lee, C., Song, Y., Conn, P. M., and Ji, T. H. (2002) *Mol Endocrinol* **16**, 1299-308.

30. Fotiadis, D., Liang, Y., Filipek, S., Saperstein, D. A., Engel, A., and Palczewski, K. (2003) *Nature* **421**, 127-8.

31. Liang, Y., Fotiadis, D., Filipek, S., Saperstein, D. A., Palczewski, K., and Engel, A. (2003) *J Biol Chem.* **278**, 21655-62.

32. Rieke, F., and Baylor, D. A. (1998) *Biophys J* **75**, 1836-57.

33. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H., Pollak, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) *J Biol Chem* **271**, 19537-45.

34. Rodriguez-Frade, J. M., Vila-Coro, A. J., de Ana, A. M., Albar, J. P., Martinez, A. C., and Mellado, M. (1999) *Proc Natl Acad Sci U S A* **96**, 3628-33.

35. Overton, M. C., and Blumer, K. J. (2000) *Curr Biol* **10**, 341-4.

36. Yesilaltay, A., and Jenness, D. D. (2000) *Mol Biol Cell* **11**, 2873-84.

37. Overton, M. C., and Blumer, K. J. (2002) *J Biol Chem* **277**, 41463-72.

38. Dohlman, H. G., and Thorner, J. W. (2001) *Annu Rev Biochem* **70**, 703-754.

39. Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998) *Mol Biol Cell* **9**, 885-99.

40. Sprague, G. F., Jr. (1991) *Methods Enzymol* **194**, 77-93.

41. Blumer, K. J., Reneke, J. E., and Thorner, J. (1988) *J Biol Chem* **263**, 10836-42.
42. Weiner, J. L., Gutierrez-Steil, C., and Blumer, K. J. (1993) *J Biol Chem* **268**, 8070-7

43. Stefan, C. J., and Blumer, K. J. (1994) *Mol Cell Biol* **14**, 3339-49

44. Celic, A., Martin, N. P., Son, C. D., Becker, J. M., Naider, F., and Dumont, M. E. (2003) *Biochemistry* **42**, 3004-17.

45. Dosil, M., Schandel, K. A., Gupta, E., Jenness, D. D., and Konopka, J. B. (2000) *Mol Cell Biol* **20**, 5321-9.

46. Reneke, J. E., Blumer, K. J., Courchesne, W. E., and Thorner, J. (1988) *Cell* **55**, 221-34

47. Parrish, W., Eilers, M., Ying, W., and Konopka, J. B. (2002) *Genetics* **160**, 429-43

48. Apanovitch, D. M., Slep, K. C., Sigler, P. B., and Dohlman, H. G. (1998) *Biochemistry* **37**, 4815-22

49. Shah, A., and Marsh, L. (1996) *Biochem Biophys Res Commun* **226**, 242-6

50. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) *Science* **289**, 739-45.

51. Monnot, C., Bihoreau, C., Conchon, S., Curnow, K. M., Corvol, P., and Clauser, E. (1996) *J Biol Chem* **271**, 1507-13

52. Gouldson, P. R., Snell, C. R., Bywater, R. P., Higgs, C., and Reynolds, C. A. (1998) *Protein Eng* **11**, 1181-93

53. Schwyzer, R. (1977) *Ann. N. Y. Acad. Sci.* **247**, 3-26

54. Carrillo, J. J., Pediani, J., and Milligan, G. (2003) *J Biol Chem* **14**, 14

55. Baneres, J. L., and Parello, J. (2003) *J Mol Biol* **329**, 815-29.
FIGURE LEGENDS

Figure 1. Models of trans activation between GPCR subunits and schematic of the α-factor receptor. (A) Allosteric, intersubunit movement and domain exchange models of trans activation between subunits in GPCR dimers/oligomers. The ligand-binding domain is designated by the trapezoid and agonist by a lightning bolt. In the allosteric transfer model, one receptor subunit binds agonist, changes conformation, thereby inducing a conformational change in the second receptor subunit that results in G protein activation. In the intersubunit movement model, agonist binding to one receptor induces movement between the two receptors, which allows the second receptor subunit to activate G proteins. In the domain exchange model, the agonist-binding domain of one receptor can active the transmembrane domains of the second receptor, leading to G protein activation. (B) Schematic of the α-factor receptor showing the amino acid sequence of TM domains and intracellular and extracellular loops. Residues indicated with black circles indicate positions of amino acid substitutions used in this study. Numbered residues are indicated with an asterisk.

Figure 2. Agonist response of cells expressing wild type and mutant receptors. The indicated receptors were expressed in cells that either expressed Sst2 (top panel) or lacked Sst2 (bottom panel).
Growth arrest assays were performed as described in “Experimental Procedures”.

Response to a single dose of agonist (α-factor; 15 nmol) is shown. Results shown are representative of 3-6 experiments for each mutant.

Figure 3. **Quantification of agonist responsiveness of cells expressing wild type and mutant α-factor receptors.** (A) Dose-response relationships indicated by assays of agonist-induced growth arrest. Cells (sst2 mutant bearing a chromosomal deletion of the receptor gene; JE114-8A) expressing tailless forms of wild type (closed circles), ic1A (open circles), 80-2A (closed squares), 151-3A (open squares), 153-5A (closed triangles) receptors from single copy plasmids and their normal promoter were assayed. Results shown for each mutant are the average of five independent experiments. (B) Dose-response relationships indicated by assays of agonist-induced expression of a FUS1-lacZ reporter. Tailless wild type and mutant receptors were expressed from a single-copy plasmid and their normal promoter in cells (KBY58) bearing a chromosomal deletion of the receptor gene and a FUS1-lacZ plasmid (pSL307). Cells expressing wild type (closed circles), ic1A (open circles), 80-2A (closed squares), 151-3A (open squares) and 153-5A (triangles) were assayed. Results for each mutant were averages of 3-4 independent experiments; s.e.m was <10% of the indicated values.

Figure 4. **Subcellular localization of wild type and mutant receptors tagged with GFP.**
Tailless forms of the indicated receptors were tagged with GFP after TM7 (residue 303) and expressed from single copy plasmids from their normal promoter in cells that carried a chromosomal deletion of the receptor gene (KBY58). Images were acquired as described in “Experimental Procedures.” The lysosome-like vacuole (V) and endoplasmic reticulum (ER) are indicated.

Figure 5. **Use of FRET to determine the efficiency of homo-oligomerization of wild type and mutant receptors in vivo.** FRET experiments were used to detect the ability of CFP- and YFP-tagged forms of the indicated tailless receptors to self-associate in living yeast cells bearing a deletion of the chromosomal receptor gene (KBY58). FRET data were collected and analyzed as described in “Experimental Procedures.” Each panel shows four emission spectra obtained upon excitation of cells at the $\lambda_{\text{max}}$ for CFP, as follows: one spectrum from cells co-expressing CFP- and YFP-tagged forms of the indicated receptor (dotted plus dashed line); a second spectrum from cells expressing only the indicated CFP-tagged receptor (dotted line); a third spectrum from cells expressing only the indicated YFP-tagged receptor (dashed line); and a fourth spectrum that shows the resultant fluorescence emission due specifically to FRET (solid line) in cells co-expressing CFP- and YFP-tagged receptors. Emission due to FRET was determined by subtracting the second and third emission spectra from the first spectrum, as described in “Experimental Procedures.” All tagged receptors were expressed from plasmids in cells carrying
a deletion of the chromosomal gene encoding the α-factor receptor. Each experiment was performed at least three times in duplicate. Results shown for each mutant are the average of four independent experiments.

Figure 6. **Use of FRET to determine the efficiency of heterooligomerization of representative mutant receptors *in vivo***. FRET experiments were used to quantify the ability of CFP- and YFP-tagged forms of the indicated tailless α-factor receptors to self-associate in living yeast cells. FRET data were collected, analyzed and quantified as described in the legend to Figure 4 and “Experimental Procedures.” Fluorescence emission due specifically to FRET is indicated by the *solid line*. Results shown for each mutant are the average of four independent experiments.

Figure 7. **Phenotypes of cells co-expressing mutant receptors determined by assays of agonist-induced growth arrest.** Growth arrest assays employing a single dose of agonist (α-factor; 15nmol) were performed as described in the legend to Figure 1. (A) Phenotypes due to individually expressed tailless receptors are shown in the top row of panels; phenotypes due to pair-wise combinations of co-expressed receptors are indicated in the lower set of panels. (B) The indicated full-length receptors were expressed individually or co-expressed together and
analyzed for function in growth assays. The relative signaling efficiency established by dose-
response experiments performed in triplicate for each cell type is indicated below each panel.

Figure 8. **Phenotypes of cells co-expressing mutant receptors determined by assays of
agonist-induced gene expression.** Dose-response relationships indicated by assays of agonist-
induced expression of a *FUS1-lacZ* reporter were determined as described in “Experimental
Procedures”. (A) Reporter gene expression in cells expressing wild type tailless receptors
(*closed circles*), ic1A mutant tailless receptors (*open circles*), 80-2A mutant tailless receptors
(*closed squares*), and co-expressing ic1A tailless receptors and 80-2A tailless receptors (*open
squares*). (B) Reporter gene expression in cells expressing wild type tailless receptors (*closed
circles*), ic1A mutant tailless receptors (*open circles*), L236R mutant tailless receptors (*closed
squares*), and co-expressing ic1A tailless receptors and L236R tailless receptors (*open squares*).
(C) Reporter gene expression in cells expressing wild type tailless receptors (*closed circles*), 80-
2A mutant tailless receptors (*open circles*), L236R mutant tailless receptors (*closed squares*), and
co-expressing 80-2A tailless receptors and L236R tailless receptors (*open squares*). (D)
Reporter gene expression in cells expressing wild type tailless receptors (*closed circles*), L236R
mutant tailless receptors (*open circles*), S184R mutant tailless receptors (*closed squares*), and co-
expressing L236R tailless receptors and S184R tailless receptors (*open squares*). For each
experiment, tailless wild type and mutant receptors were expressed from single-copy plasmids
and their normal promoter in cells (JE114-8A) bearing a chromosomal deletion of the receptor gene, an sst2-1 mutation (RGS homolog), and a plasmid (pSL307) bearing an agonist-inducible reporter (FUS1-lacZ). Results were the average of 4-6 independent experiments performed in duplicate; s.e.m. was <10% of the indicated values.

Figure 9. Pheromone response of cells expressing intragenic double mutant receptors. Full-length forms of the indicated receptors were expressed from single copy plasmids from their normal promoter in an sst2 mutant strain that carried a deletion of the chromosomal gene encoding the α-factor receptor (JE114-8A). Cells were assayed for the magnitude of growth arrest responses elicited by a single dose of agonist (α-factor; 15 nmol).
Table 1. **Agonist binding assays of cells expressing wild type or mutant α-factor receptors.** Radioligand binding assays were performed using $[^{35}\text{S}]\alpha$-factor and intact, inviable cells expressing the indicated STE2 alleles from single-copy plasmids in a strain (KBY58) carrying a deletion of the chromosomal receptor gene. All $K_d$ and cell surface expression ($B_{\text{max}}$) values were calculated by nonlinear least-squares regression of data obtained from two to six independent transformants assayed in triplicate.

| Receptor       | $K_d$ (nM) | $B_{\text{max}}$ (sites/cell) |
|----------------|------------|-------------------------------|
| wild type      | 5.4        | 10,600                        |
| wild type $\Delta$tail | 4.3        | 35,000                        |
| ic1A           | 2.5        | 2400                          |
| 80-2A          | 1.7        | 1300                          |
| 151-3A         | 1.25       | 4400                          |
| 153-5A         | 8.8        | 4300                          |
| ic1A$\Delta$tail | 0.57      | 4400                          |
| 80-2A$\Delta$tail | 0.19      | 3600                          |
| 151-3A$\Delta$tail | 0.54      | 34,000                        |
| 153-5A$\Delta$tail | 2.2       | 32,000                        |
| L236R          | 0.8        | 4000                          |
| L236R+ic1A     | 2.5        | 2000                          |
| L236R+80-2A    | n.d.       | n.d.                          |
| L236R+151-3A   | 1.2        | 2200                          |
| L236R+153-5A   | 4.8        | 2300                          |
| ic1A+151-3A    | n.d.       | n.d.                          |
| ic1A+153-5A    | n.d.       | n.d.                          |
| 80-2A+151-3A   | n.d.       | n.d.                          |
| 80-2A+153-5A   | 1.3        | 1500                          |

n.d.-specific binding not detected
Table 2. **Apparent efficiencies of homo- and heterooligomerization of wild type and mutant receptors indicated by FRET.** The ability of the indicated pairs of co-expressed YFP- and CFP-tagged tailless receptors to interact was quantified by calculating apparent FRET efficiencies. This involved dividing the peak value of the FRET spectrum by the peak value of the YFP emission spectrum obtained by exciting cells co-expressing CFP- and YFP-tagged receptors at the $\lambda_{\text{max}}$ of YFP, as described in “Experimental Procedures”; dividing the integrated areas of these two curves gave identical results. Data shown are the average of 4 experiments; standard deviations are indicated.

| Co-expressed tailless receptors | FRET efficiency (%) |
|-------------------------------|---------------------|
| **homooligomerization**       |                     |
| wild type + wild type         | 9.4 ± 0.2           |
| ic1A + ic1A                   | 13.2 ± 1.9          |
| TM2-80-2A + TM2-80-2A         | 8.4 ± 0.2           |
| TM3-151-3A + TM3-151-3A       | 8.4 ± 0.7           |
| TM3-153-5A + TM3-153-5A       | 9.1 ± 0.5           |
| L236R + L236R                 | 9.7 ± 0.6           |
| S184R + S184R                 | 9.5 ± 0.4           |
| **heterooligomerization**     |                     |
| L236R + TM3-151-3A            | 8.3 ± 0.6           |
| L236R + TM3-153-5A            | 9.4 ± 0.7           |
| S184R + L236R                 | 9.2 ± 0.6           |
| S184R + TM3-151-3A            | 8.1 ± 0.4           |
| S184R + TM3-153-5A            | 8.3 ± 0.2           |
| n.d.-not detected             |                     |
Table 3. Quantification of agonist sensitivities of cells expressing mutant receptors individually versus cells co-expressing pairwise combinations of mutant receptors in trans. Agonist sensitivities were quantified by performing dose-response assays of α-factor-induced growth arrest, as described in “Experimental Procedures”. The host strain (JE114-8A) used for receptor expression from single-copy plasmids carried a deletion of the chromosomal gene encoding the α-factor receptor, and an sst2-1 mutation (RGS homolog) to inhibit desensitization and thereby yield clear zones of growth inhibition that could be measured accurately. Data shown are the average of at least four assays each performed in triplicate; s.e.m. is indicated.

| Tailless receptors expressed individually | Agonist sensitivity (relative to wild type tailless receptor expressed alone) | Sensitivity upon co-expression | Sensitivity expressed alone |
|-----------------------------------------|-------------------------------------------------|-----------------------------|-----------------------------|
| wild type                               | 1.0 ± 0.3                                        | n.a.                        |                             |
| ic1A                                    | 0.05 ± 0.002                                     | n.a.                        |                             |
| 80-2A (endofacial TM2)                  | 0.11 ± 0.01                                      | n.a.                        |                             |
| 151-3A (endofacial TM3)                 | 0.13 ± 0.01                                      | n.a.                        |                             |
| 153-5A (endofacial TM3)                 | 0.06 ± 0.002                                     | n.a.                        |                             |
| L236R (ic3)                             | 0.02 ± 0.005                                     | n.a.                        |                             |
| S184R (exofacial TM4)                   | 0.005 ± 0.002                                    | n.a.                        |                             |

| Tailless receptors co-expressed          | Agonist sensitivity | Sensitivity upon co-expression | Sensitivity expressed alone |
|-----------------------------------------|---------------------|--------------------------------|-----------------------------|
| S184R + ic1A                            | 0.13 ± 0.03         | 2.6 (ic1A)                     |                             |
| S184R + 80-2A                           | 0.20 ± 0.01         | 1.8 (80-2A)                    |                             |
| S184R + 151-3A                          | 0.14 ± 0.005        | 1.1 (151-3A)                   |                             |
| S184R + 153-5A                          | 0.08 ± 0.01         | 1.3 (153-5A)                   |                             |
| S184R + L236R                           | 0.03 ± 0.005        | 1.5 (L236R)                    |                             |
| L236R + ic1A                            | 0.30 ± 0.04         | *6.0 (ic1A)                    |                             |
| L236R + 80-2A                           | 0.33 ± 0.04         | *3.0 (80-2A)                   |                             |
| L236R + 151-3A                          | 0.22 ± 0.005        | 1.7 (151-3A)                   |                             |
| L236R + 153-5A                          | 0.13 ± 0.03         | 2.2 (153-5A)                   |                             |
| 153-5A + ic1A                           | 0.15 ± 0.02         | 2.5 (153-5A)                   |                             |
| 153-5A + 80-2A                          | 0.24 ± 0.01         | 2.2 (80-2A)                    |                             |
| 153-5A + 151-3A                         | 0.15 ± 0.01         | 1.2 (151-3A)                   |                             |
| 151-3A + ic1A                           | 0.16 ± 0.05         | 1.2 (151-3A)                   |                             |
| 151-3A + 80-2A                          | 0.22 ± 0.02         | 2.0 (80-2A)                    |                             |
| 80-2A + ic1A                            | 0.19 ± 0.01         | 1.7 (80-2A)                    |                             |

| Full-length receptors                    | Agonist sensitivity | Sensitivity upon co-expression | Sensitivity expressed alone |
|-----------------------------------------|---------------------|--------------------------------|-----------------------------|
| wild type                               | 1.0 ± 0.03          | n.a.                           |                             |
| S184R                                   | 0.03 ± 0.002        | n.a.                           |                             |
| L236R                                   | 0.16 ± 0.03         | n.a.                           |                             |
| S184R + L236R                           | 0.17 ± 0.08         | 1.1 (L236R)                    |                             |
n.a.-not applicable. *mutant pairs where co-expression improved agonist sensitivity at least 3-fold relative to cells expressing one of the corresponding pair of receptors that had the greater function (indicated in parenthesis).
Table 4. **Quantification of agonist sensitivities of cells expressing mutant receptors individually versus cells co-expressing mutant receptors in trans.** Agonist sensitivities were quantified by performing dose-response assays of α-factor-induced expression of a *FUS1-lacZ* reporter, as described in “Experimental Procedures”. The host strain (JE114-8A) used for receptor expression from single-copy plasmids carried a deletion of the chromosomal gene encoding the α-factor receptor (*STE2*), and an *sst2-1* mutation that inactivates an RGS homolog that negatively regulates signaling. Results of 4-6 assays performed in duplicate are shown.

| Tailless receptors expressed individually | Agonist Sensitivity (relative to wild type tailless receptor) | Sensitivity upon co-expression Sensitivity expressed alone |
|------------------------------------------|-------------------------------------------------------------|-----------------------------------------------------------|
| wild type                                | 2.0 (nM)                                                   | 1.0 n.a.                                               |
| ic1A                                     | 24 (nM)                                                   | 0.08 n.a.                                              |
| 80-2A                                    | 14 (nM)                                                   | 0.14 n.a.                                              |
| L236R                                    | 180 (nM)                                                  | 0.01 n.a.                                              |
| S184R                                    | 900 (nM)                                                  | 0.002 n.a.                                             |
| Tailless receptors co-expressed           |                                                             |                                                          |
| ic1A + 80-2A                              | 3.0 (nM)                                                  | 0.7 5.0 (80-2A)                                         |
| ic1A + L236R                              | 3.0 (nM)                                                  | 0.7 8.7 (ic1A)                                          |
| 80-2A + L236R                             | 1.7 (nM)                                                  | 1.2 8.6 (80-2A)                                         |
| L236R + S184R                             | 130 (nM)                                                  | 0.02 2.0 (L236R)                                        |

n.a.-not applicable.
Figure 2

|              | +Sst2 |                  |                  |                  |                  |
|--------------|-------|------------------|------------------|------------------|------------------|
|              | WT    | ic1A             | 80-2A            | 151-3A           | 153-5A           |
| Full-length  |       |                  |                  |                  |                  |
| Δtail        |       |                  |                  |                  |                  |
| Over-expressed Δtail |   |                  |                  |                  |                  |

|              | -Sst2 |                  |                  |                  |                  |
|--------------|-------|------------------|------------------|------------------|------------------|
|              |       |                  |                  |                  |                  |
| Full-length  |       |                  |                  |                  |                  |
| Δtail        |       |                  |                  |                  |                  |
| Over-expressed Δtail |   |                  |                  |                  |                  |
Figure 4

ic1A

80-2A

151-3A

153-5A

Wild type
Figure 5
Figure 6

Emission Intensity (arbitrary units)

wavelength (nm)
Figure 7

A

Individual tailless receptors

| WT | ic1A | 80-2A | 151-3A | 153-5A | L236R | S184R |
|----|------|-------|--------|--------|-------|-------|

Tailless co-expressed receptors

| ic1A | 80-2A | 151-3A | 153-5A | L236R |
|------|-------|--------|--------|-------|
| S184R|
| L236R|
| 153-5A|
| 151-3A|
| 80-2A |

B

Full-length receptors

| WT | S184R | L236R | S184R + L236R |
|----|-------|-------|----------------|

Downloaded from http://www.jbc.org/ by guest on March 25, 2020
Figure 8

A

Beta-galactosidase Activity (Miller Units)

log[alpha-factor (M)]

B

Beta-galactosidase Activity (Miller Units)

log[alpha-factor (M)]

C

Beta-galactosidase Activity (Miller Units)

log[alpha-factor (M)]

D

Beta-galactosidase Activity (Miller Units)

log[alpha-factor (M)]
Figure 9
Subunits of a yeast G protein-coupled receptor are activated independently by agonist but function in concert to activate G protein heterotrimers
Sharon L. Chinault, Mark C. Overton and Kendall J. Blumer

J. Biol. Chem. published online February 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311099200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts