Oligomerization of the Yeast α-Factor Receptor

IMPLICATIONS FOR DOMINANT NEGATIVE EFFECTS OF MUTANT RECEPTORS*

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Oligomerization of G protein-coupled receptors is commonly observed, but the functional significance of oligomerization for this diverse family of receptors remains poorly understood. We used bioluminescence resonance energy transfer (BRET) to examine oligomerization of Ste2p, a G protein-coupled receptor that serves as the receptor for the α-mating pheromone in the yeast Saccharomyces cerevisiae, under conditions where the functional effects of oligomerization could be examined. Consistent with previous results from fluorescence resonance energy transfer (Overton, M. C., and Blumer, K. J. (2000) Curr. Biol. 10, 341–344), we detected efficient energy transfer between Renilla luciferase and a modified green fluorescent protein individually fused to truncated α-factor receptors lacking the cytoplasmic C-terminal tail. In addition, the low background of the BRET system allowed detection of significant, but less efficient, energy transfer between full-length receptors. The reduced efficiency of energy transfer between full-length receptors does not appear to result from different levels of receptor expression. Instead, attenuation of fluorescent reporter proteins to the full-length receptors appears to significantly increase the distance between reporters. Mutations that were previously reported to block dimerization of truncated α-factor receptors reduce but do not completely eliminate BRET transfer between receptors. Dominant negative effects of mutant alleles of α-factor receptors appear to be mediated by receptor oligomerization since these effects are abrogated by introduction of additional mutations that reduce oligomerization. We find that heterodimers of normal and dominant negative receptors are defective in their ability to signal. Thus, signal transduction by oligomeric receptors appears to be a cooperative process requiring interaction between functional monomers.

G protein-coupled receptors (GPCRs)3 comprise a large family of cellular receptors responsible for transducing signals from a wide variety of extracellular stimuli including peptides, neurotransmitters, hormones, and light. All GPCRs are transmembrane proteins consisting of an extracellular N-terminal domain, seven transmembrane α-helical segments, and a cytoplasmic C-terminal tail. A large body of evidence indicates that GPCRs form homo- and/or hetero-oligomeric complexes in cells (2–5). Although the implications of oligomerization for receptor function remain poorly understood, in some cases oligomerization is capable of affecting biogenesis and membrane targeting of receptors (6, 7). In addition, cooperation between different monomers appears to be responsible for mediating or modulating the signaling function of some GPCRs (8, 9).

The possibility of artifactual aggregation (or dis-aggregation) of GPCRs during solubilization and extraction from the membranes makes it desirable to monitor the oligomeric state of receptors that are maintained in their native cellular membranes. Because detection of nonradiative energy transfer between fluorescent or bioluminescent probes provides an assay for oligomerization that can be readily performed with proteins in cells or in membrane fractions, fluorescence resonance energy transfer (FRET) and bioluminescence energy transfer (BRET) have been used to monitor homo- and hetero-oligomerization of GPCRs (1, 10–15). An alternative approach for detecting oligomerization of receptors in living cells has been the detection of altered intracellular trafficking of receptors resulting from co-expression of receptors with different trafficking behaviors (16–20).

The α-mating pheromone receptor, Ste2p, is a GPCR that activates the pheromone response pathway of the yeast Saccharomyces cerevisiae. The MATα and MATα haploid cell types of S. cerevisiae secrete the peptide mating pheromones a-factor and α-factor, respectively. Upon binding α-factor, the α-factor receptor expressed on the surface of MATα cells causes exchange of GTP for GDP on a cytoplasmic heterotrimeric G protein. Release of the Gβγ subunits, encoded by the STE4 and STE18 genes, from the GTP-bound Gα subunit, encoded by the GPA1 gene, leads to activation of a mitogen-activated protein kinase cascade. This results in cell cycle arrest, transcriptional activation of many genes, and other physiological responses that prepare cells for mating.

In a number of situations in different cell types, expression of one type of receptor or a mutant form of a receptor can affect signaling by a different type of receptor allele co-expressed in green fluorescent protein; GFPβ, GFP optimized for BRETβ; RLuc, Renilla reniformis luciferase; DBC, DeepBlueCTM commercially available coelenterazine derivative; HA, hemagglutinin; PBS, phosphate-buffered saline.
the same cell type (21–24). In yeast, co-expression of functional and defective alleles of Ste2p can result in a decrease in signaling by the functional allele (25, 26), and co-expression of normal and constitutively active receptor alleles causes suppression of constitutive signaling (27). Both receptor oligomerization and competition for access to a substoichiometric amount of G protein have been proposed as explanations for these dominant effects (1, 26). Although oligomerization of normal α-factor receptors has been demonstrated using FRET (1), co-immunoprecipitation (17), and co-sequstration of different STE2 alleles in endocyted membranes (16), to date there has been no direct demonstration of co-oligomerization of normal and dominant negative alleles or of normal and constitutive alleles in cells. Thus, the role of oligomerization in explaining the dominant effects of STE2 alleles has not been established. In fact, the question of whether the association of GPCRs into oligomers has a direct role in signaling function remains unresolved in most GPCR systems. A major barrier to determining the functional role of oligomerization in the yeast system is that it has only previously been possible to detect FRET transfer between truncated receptors lacking C-terminal cytoplasmic tails (1), whereas removal of the tails diminishes or eliminates dominant negative effects of mutant receptors (28). Removal of the tail from normal receptors does not interfere with signaling and, in fact, enhances sensitivity to pheromone (29, 30).

We report here that BRET can be used in yeast cells to detect oligomerization of α-factor receptors with a sensitivity that allows detection of oligomers of full-length receptors. Full-length receptors exhibit a decrease in energy transfer efficiency compared with C-terminal-truncated receptors that can be explained by increased distance between C termini of full-length receptors. The ability to detect oligomerization of full-length receptors made it possible to demonstrate co-oligomerization of dominant negative and normal receptor alleles. Dominant negative behavior of mutant receptors appears to stem from an inability to signal on the part of normal receptors that co-oligomerize with defective receptors. In agreement with a growing literature from other systems (31–33), these results indicate that individual receptors that are components of oligomers do not act independently.

MATERIALS AND METHODS

Strains and Plasmids (See Tables 1 and 2)—The S. cerevisiae host strain used for signaling assays and ligand binding was A230 (MATa cyr5 ade2–1 his4–580 lys2–oc trp1–am tyr1–oc SUP4–3′ leu2 ura3 bar1–1), carrying a mutation of the BAR1 gene that encodes a protease for A-factor. Strain A232 (25), which has the same genetic background as A230 but also carries a chromosomal deletion of the α-factor receptor gene (STE2), was used as a host for monitoring BRET of truncated receptors and for initial BRET experiments with full-length receptors. To reduce intracellular proteolysis of fusion proteins, the S. cerevisiae strain A3102 (MATa pep4–3 his4–580 ura3–52 leu2–3, 112 ste2–Δ) carrying a deletion of the proteasine A gene, PEP4, was used for most energy transfer assays with full-length Ste2p receptors. To create A3102, A3087 (MATa pep4–3 his4–580 ura3–52 leu2–3, 112) (34) was transformed with pMD147 digested with PmlI followed by transfer to 5-fluoroorotic acid to delete STE2 (as previously described (25)). Strain A2638 (MATa leu2 trp1 ura3–52 prb1–1122 pep4–3 Δhis3pGAL10-GAL4) (35), provided by Dr. Elizabeth Grayhack, was used for BRET experiments with positive and negative control constructs expressed under GAL control. All transformations were accomplished using the one-step procedure of Chen et al. (36). All strains containing two different plasmids were generated by sequential transformation of the constructs into the host strain to avoid homologous recombination.

Full-length fusions of Ste2p with BRET partners were expressed from plasmids derived from a multicopy, STE2-encoding plasmid pMD240 (25). As an initial step toward creating the fusions, plasmid pMD1003 was constructed by site-directed mutagenesis using oligonucleotide ON504 (sequences of oligonucleotides are available upon request) to introduce an Xhol site 3′ to the influenza hemagglutinin (HA) epitope tag at the C-terminal of the STE2 gene in pMD240. C-terminal-truncated alleles of Ste2p were derived from plasmid pMD626 described previously (37) containing residues 1–304 of the receptor. Plasmid pMD803 was created via KpnI and SacI digest of pMD626 and insertion into pMD240 to create a c-Myc-tagged truncated Ste2p receptor. An Nhel-SacI fragment of pMD626 containing the C-terminal region of the truncated receptor was also ligated into Nhel- and SphI-digested pMD240 to create pMD985. An Xhol site was also introduced into pMD985 immediately 3′ to a c-Myc epitope tag by site-directed mutagenesis using ON512, creating plasmid pMD1055. All plasmids constructed from pMD985 contain a Cys-252→Ser mutation that does not affect receptor function (38).

GFP2 was amplified by PCR using oligonucleotides ON591 and ON592 and using plasmid pGFP2-RLuc(h)TM (PerkinElmer Life Sciences) as a template. RLuc was amplified from the same template using oligonucleotides ON616 and ON617. In each case, the primers added Sall sites flanking the protein reading frame. The PCR products were cloned into pCR®-Blunt II® TOPO® vector (Invitrogen) to generate pMD1120 and pMD1121 containing GFP2 and RLuc, respectively. The BRET proteins were excised from these plasmids using Sall and inserted into the compatible Xhol site that had been introduced into full-length Ste2p in pMD1003, creating plasmid pMD1132 (containing GFP2) and pMD1133 (containing RLuc). Fusions to truncated Ste2p were created similarly by inserting into Xhol-cut pMD1055, creating plasmids pMD1134 (containing GFP2) and pMD1135 (containing RLuc). All RLuc constructs were also subcloned into vectors containing auxotrophic LEU2 markers to allow co-expression in strains. SacI-Sphl fragments containing STE2–RLuc fusions were ligated into the multicopy LEU2 vector pMD107 (YEplac181) (39) to generate pMD1137 (Ste2p–RLuc) and pMD1139 (truncated Ste2p–RLuc). Epitope tags were removed from RLuc and GFP2 constructs by digesting with NotI and re-ligating to generate pMD1167 (Ste2p–GFP2), pMD1168 (Ste2p–RLuc), pMD1154 (truncated Ste2p–GFP2), and pMD1155 (truncated Ste2p–RLuc).

To allow expression of BRET fusions at lower levels, they were also transferred to CEN (centromere-containing) plasmids (see Table 1). Plasmid pMD149 (25) was digested with SacI and Sphl so that the STE2 that was originally present could
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be replaced by a SacI-SphI fragment from pMD1154 to generate pMD1198 containing truncated Ste2p-GFP2. A SacI-SphI cut insert from pMD1167 was then ligated into pMD1198 to generate pMD1242, containing Ste2p-GFP2. An EcoRI-SphI cut insert from pMD1155 was ligated into pMD836, YCplac111 (39), to generate pMD1199 containing truncated Ste2p-Rluc. Also, an EcoRI-SphI cut insert from pMD1168 was ligated into pMD836 to generate pMD1232, containing Ste2p-Rluc.

An internal XhoI site for fusion of BRET reporters internally into the STE2 ORF was introduced by PCR amplification of the C-terminal region of a wild type STE2 from pMD240 with ON786 that introduces an XhoI site immediately 3’ to an NsiI site in the STE2 gene and ON787. The PCR product was digested with BsrGI and NsiI and subcloned into similarly cleaved pMD149 to generate pMD1203. Plasmid pMD1203 was then digested with SacI and SphI and subcloned into both pMD107 and pMD240 to produce pMD1206 (LEU2) and pMD1207 (URA3), respectively. GFP2 and Rluc were inserted into the introduced XhoI sites in these plasmids as described above, except that they were amplified using 3’ primers ON802 and ON803, respectively, which removed their endogenous termination codons. Rluc was inserted into XhoI-cut pMD1206 to produce pMD1212, whereas GFP2 was inserted into XhoI-cut pMD1207 to produce pMD1211.

In some constructs the C-terminal tail (residues 305–431) of Ste2p was replaced by an unrelated C-terminal tag containing His6 and ZZ domains derived from a vector used for the yeast MORF expression library (34). To create constructs in which the BRET reporters were internally fused between the STE2 gene and this exogenous tail, the tag region extending from the His6 tag to exactly 400 bp 3’ of the stop codon derived from BG1805 (provided by Elizabeth Grayhack) was amplified using primers ON823 and ON824 to introduce NotI and SacI sites for Gateway recombinational cloning (Invitrogen). The PCR product was digested with SphI and NotI and subcloned into similarly cut pMD107 to create the STE2 ORF with pMA1 to create pMD1209.

A fusion of GFP2 and Rluc that could be expressed in yeast was created by PCR amplification of the insert from the pGFP2: Rluc(h)TM positive control construct (PerkinElmer) using primers ON818 and ON819 that flank GFP2-Rluc with AttB sites for Gateway recombinational cloning (Invitrogen). The two-step reaction for cloning into destination vector BG1805 for expression under GAL control was conducted as described by Gelperin et al. (34) to create pMD1144 (see Table 1). GFP2 alone was cloned similarly into the same vector after PCR amplification with primers ON618 and ON619 to create pMD1146. A SacI-Asp718 fragment containing GFP2 was subcloned from pMD1146 into SacI-Asp718-cut pMD107 to create pMD1148 with a LEU2 marker. Rluc alone was subcloned into BG1805 after PCR with primers ON644 and ON619 to create pMD1147.

To create GFP2-Rluc fusion constructs connected by linkers of varying lengths, a Spel site was introduced between the two reading frames in pMD1144 via site-directed mutagenesis with ON852 to create pMD1301. A triple tandem repeat of an HA epitope (clone 12CA5) was PCR-amplified from pMD240 with ON849 and ON850 flanking the tag with Nhel and Spel sites, respectively. The PCR product was subcloned into pMD1301 via Nhel/Spel digest to create pMD1303. The retention of a single Spel site in the plasmid allowed for a repeated cycle of the same subcloning procedure to generate pMD1304 containing up to six repeats of the HA epitope tag.

Dimerization-defective receptors were constructed by site-directed mutagenesis of plasmid pMD240 with oligonucleotides ON809 and ON810 to create pMD1217, containing G56A/G60A mutations, and pMD1218, containing G56L/G60L mutations, respectively. Splh-Nhel fragments from pMD1217 and pMD1218 were used to replace the corresponding wild-type sequences by ligating into Splh-Nhel-cut pMD1154 to create GFP2-tagged truncated receptor constructs pMD1224 and pMD1225, respectively. Splh-KpnI fragments from pMD1217 and pMD1218 were cloned as replacements for wild-type sequences by ligating into Splh-KpnI-cut pMD1155 to create GFP2-Nhel-luc fusion constructs containing the glycine mutations, pMD1217 and pMD1218 were digested with KpnI and subcloned into pMD1119 to create pMD1381 and pMD1382, respectively. To create CEN constructs containing the glycine mutations, pMD1217 and pMD1218 were digested with Splh/KpnI and subcloned into pMD1199 to create pMD1381 and pMD1382, respectively.

To create dominant negative receptors, the plasmid pMD294 (25) containing the Y266C mutation was digested with Splh and Asp718 and subcloned into pMD1154 to generate pMD1173. To make full-length Ste2p Y266C-GFP2 mutant receptors, an Asp718-Splh fragment was subcloned from pMD1173 into pMD1167 to generate pMD1244 (see Table 1). To make dimerization-defective dominant receptors that also contain the Y266C mutation, a SacI-Nhel fragment from pMD1244 was ligated into pMD1235 to generate pMD1263. To make the dominant negative mutant Y266C without BRET tags, pMD240 and pMD149 were altered by site-directed mutagenesis with oligonucleotide ON51 to create pMD294 and
F204S-GFP2, pMD1205 was sequentially digested with SphI to create pMD1206. To create Ste2p ON404 was used to introduce F204S into pMD240 via site-directed mutagenesis to create pMD1205. To create Ste2p, sites were cut by SphI and SalI and subcloned into pMD1244 to create pMD1414 (see Table 1). To create dimerization-defective F204S receptors, pMD1217 was digested with StuI and SphI and subcloned into pMD1409 to create pMD1410. Finally, SacI-SphI fragments from pMD1205 was inserted to create pMD1407. Then, pMD1218 was digested with StuI and SacI and inserted into pMD1407 to create pMD1408. Finally, SacI-SphI fragments from pMD1407 and pMD1408 were subcloned into pMD228 (25) to create pMD1409 and pMD1410, respectively (see Table 2).

Bioluminescence Resonance Energy Transfer—In preparation for BRET measurements, cells were picked from plates (SD – Ura – Leu media) (40) and cultured overnight in 4 ml of liquid SD – Ura – Leu media. Cultures were then diluted and grown to an A600 of 1. A total of 3 ml of culture was centrifuged at 13,000 × g for 5 min. The pellet was resuspended in 200 μl of BRET buffer that contained 0.01% (w/v) magnesium (MgSO4·7H2O) and 0.1% (w/v) glucose in phosphate-buffered saline (PBS) (0.14 M NaCl 10 mM NaH2PO4, pH 7.4). Cells were recentrifuged at 13,000 × g for 3 min, the supernatants were decanted, and the pelleted cells were placed on ice. For freeze-thaw treatment, cell pellets were placed in a dry ice/isopropanol bath for 3 min then immediately transferred to a room temperature bath for another 3 min. The cell pellets were then resuspended in 200 μl of BRET buffer and inserted into a Quantamaster spectrofluorometer (Photon Technology International). Dark noise was monitored at 390 and 515 nm (25-nm bandpass), with a 1-s integration time for ~90 s using the multiwavelength detection application Felix (Photon Technology International). A 1 mM stock concentration of the Rluc substrate, the coelenterazine derivative DeepBlueC11 (DBC) (PerkinElmer) in anhydrous ethanol, was diluted 1:100 in 200 μl of BRET buffer, briefly vortexed, then mixed with the sample in the cuvette for a final concentration of 5 μM (as recommended by PerkinElmer). Data collection was resumed as quickly as possible at 390 and 515 nm (25-nm bandpass), with a 1-s integration time.

For membrane preparation of cells for BRET measurements, a modified procedure based on that of Konopka and co-workers (41) as adapted by our laboratory, was used.2 Approximately 30 ml of cells were cultured to an A600 of 1, then harvested by centrifugation at 4°C for 5 min at 12,000 × g. Pellets were resuspended in 1 ml of PBS, transferred to 2-ml microcentrifuge tubes (Laboratory Product Sales), and centrifuged again at 13,000 × g for 3 min at 4°C. Pellets were resuspended in 1 ml of PBS buffer supplemented with 2 mM Pefabloc (Roche Applied Science) and 5 μM pepstatin A (Roche) and mixed with ~250 μl of 0.5-mm zirconia/silica beads (Biospec Products). Cells were broken by five cycles of vortexing for 1 min at 4°C followed by incubation for 1 min on ice. Samples were then centrifuged for 1 min at 100 × g to remove beads and unbroken cells. The supernatant was transferred to a fresh microcentrifuge tube and centrifuged at 13,000 × g for 45 min at 4°C. The pellet from this spin was resuspended directly into 200 μl of BRET buffer and assayed for BRET as described above.

BRET ratios were calculated as follows. Background noise collected at 390 nm (Rluc) and 515 nm (GFP2) was averaged over time (~90 s) before DBC addition. After the addition of substrate, both wavelengths were alternately monitored for emission. The average emission for each wavelength before DBC injection was subtracted from each time point after injection for the corresponding wavelength. The sum of the first two corrected values at 515 nm was divided by the sum of the first two corrected values at 390 nm. An apparent ratio calculated in this way for control strains expressing Rluc alone was subtracted as a background correction from the ratio for experimental strains expressing both Rluc and GFP2. The BRET ratios presented represent the averages ± S.E. of the mean for three independent experiments (ratios were multiplied by 100 for expression as percentages). Rluc emission decays rapidly with time (42, 43). BRET ratios monitored as a function of time after the addition of substrate remained constant within ~20% for as long as the signal remained detectable over background fluctuations.

Liquid Assays of FUS1-lacZ Induction—β-Galactosidase assays of the FUS1-lacZ induction were performed as described previously (27, 44). Cells were incubated for 105 min with α-factor. Error bars indicate the S.E. of the mean for three independent isolates of the same strain.

Fluorescent (Lys7(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD), Norleucine12)-α-Factor Binding to Yeast Cells—Preparation of ligand solutions and yeast cultures for equilibrium binding analysis was performed as described previously (38). For determination of the binding affinities and numbers of sites for different yeast strains, measurements were made at concentrations of α-factor up to 600 nM.

Immunodetection of Receptors—For determination of cellular levels of receptor expression, relevant strains were cultured to an A600 of 1 in SD – Ura – Leu media. A total of 4 × 107 cells was harvested by centrifugation at 13,000 × g for 3 min, resuspended in 25 mM Tris-HCl, pH 8.0, and recentsrifuged. Pelleted cells were resuspended to 2 × 106 cells/ml in 40 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, 5% SDS, 9 M urea, 5% β-mercaptoethanol, and 200 μM Pefabloc (Roche Applied Science). Approximately 0.15 g of 0.5-mm zirconia/silica beads were added, and the suspensions were vortexed at 4°C for 5 min. Samples were then centrifuged for 10 min at 13,000 × g, and the supernatant was incubated at 37°C for 5 min. 5 μl of supernatant was loaded onto 12% SDS-polyacrylamide gels. Protein was transferred to nitrocellulose filters overnight. Filters were then blocked with PBS supplemented with 0.1% Tween and 5% newborn calf serum for 2 h. After two washes with PBS and 0.1% Tween, filters were incubated with 1:10,000 mouse-anti-GFP antibodies (Roche Applied Science), 1:10,000 mouse-anti-Rluc antibodies (Chemicon) or 1:10,000 rat-anti-HA antibodies.
(Roche) in PBS and 5% serum for 60 min. Due to low expression of GFP2-Rluc fusions with linkers (Fig. 5D), probing required 1:1000 mouse-anti-Rluc. The filters were then washed 5 times with PBS containing 0.1% Tween and 5% serum, incubated for 60 min with 1:10,000 horseradish peroxidase-conjugated goat anti-mouse or anti-rat antibodies (Bio-Rad) in PBS/Tween/serum, then washed 4 times with PBS containing 0.1% Tween. ECL Plus (Amersham Biosciences) was used for chemiluminescent detection of receptors.

RESULTS

BRET in Yeast Cells—Previous applications of FRET for detecting oligomerization of α-factor receptors in yeast required the use of significant computational and experimental correction procedures to remove contributions of incident illumination to direct acceptor activation and overlap between donor and acceptor emission spectra (1). The difficulty of applying such corrections at low transfer efficiencies may have been responsible for the reported inability of Overton and Blumer (1) to detect FRET between full-length receptors. Because previously reported dominant negative interactions between co-expressed receptor alleles in yeast could only be detected with full-length receptors (28), we investigated whether the use of BRET as an alternative to FRET might enhance sensitivity to a level adequate for detecting additional receptor-receptor interactions. BRET is based on energy transfer to an acceptor from a bioluminescent luciferase donor and, thus, requires no illuminating light source. The lack of incident illumination and the large spectral shift in the BRET2 system between the bioluminescent emission (390 nm) and the fluorescence emission of the GFP2 acceptor (515 nm) (45) were expected to lead to higher sensitivity and less need for corrections compared with FRET. An earlier version of BRET has been reported to be 10-fold more sensitive than FRET (46).

Energy transfer was readily detected between Rluc and a GFP2 acceptor to which it is covalently fused in a positive control construct, expressed under control of the GAL1 promoter (Fig. 3). The uncorrected level of GFP2 emission from this strain at 515 nm was a little more than 20% of the level of bioluminescent emission from Rluc (See Fig. 1). A particular concern was whether the substrate for the luciferase, DBC, could efficiently enter yeast cells. Fig. 2A shows bioluminescent emission at 390 nm of a yeast strain expressing a Ste2p-Rluc fusion. Bioluminescent emission was measured in a spectrofluorometer with the light source turned off. The addition of DBC to intact cells leads to only a slight increase in Rluc emission at 390 nm. Pretreatment of the cells with a cycle of freezing and thawing apparently increased permeability to DBC (Fig. 2B), leading to a sharp increase in Rluc emission followed by immediate decay of signal. The transient nature of the bioluminescent signal is a general feature of BRET2 in many systems (42) and appears to be concentration-independent as higher doses of DBC elicited a response with similar decay kinetics (data not shown). The strength of the bioluminescent signal could be further increased 10-fold using membrane preparations from yeast cells (Fig. 2C). The additional increase is apparently the result of achieving higher concentrations of protein in the cuvette (without significantly increasing scattering) and further removing barriers to access of substrate to Rluc. For reasons that are not yet clear, the BRET2 ratio observed in a membrane preparation of a strain is generally slightly lower than the ratio observed for freeze-thawed cells of the same strain. Thus, in the figures associated with this manuscript, we present only comparisons of BRET2 ratios obtained using similar preparations of relevant strains.
Rluc and GFP2 have no intrinsic tendency to interact when co-expressed in yeast. Expression of high levels of Rluc and GFP2 in the co-expressing strain was evident from the high level of Rluc bioluminescence observed, from fluorescent emission of GFP2 upon excitation in a spectrofluorometer at 410 nm (data not shown), and from immunoblotting with anti-GFP antibodies (Fig. 3, inset).

Homodimerization of Full-length and Truncated α-Factor Receptors—To monitor dimerization of the Ste2p receptor, we created constructs in which Rluc and GFP2 were each individually fused to the C termini of full-length and C-terminal-truncated (1–304) receptors (Fig. 1 and Table 1). The C-terminal tail of Ste2p is involved in receptor desensitization but is dispensable for signaling (28–30). Furthermore, it has been previously shown that fusion of fluorescent proteins to the C terminus of truncated Ste2p does not disrupt binding affinity, surface targeting, or signaling efficiency (47). In agreement with previous detection of FRET between truncated Ste2p receptors, we observed a BRET ratio of 16% for transfer from Rluc-tagged truncated Ste2p receptors to GFP2-tagged truncated Ste2p receptors in freeze-thawed cells. This ratio was almost as high as that observed for soluble covalent Rluc-GFP2 fusions, indicative of a strong tendency to dimerize (Fig. 4A). Formation of a triply fused construct by attachment of covalently fused Rluc-GFP2 to truncated Ste2p resulted in a BRET of about 30%, even higher than the ratio observed for soluble Rluc-GFP2 (data not shown). This elevated ratio is likely to be the result of Ste2p-mediated dimerization of the Rluc-GFP2 fusions, allowing energy transfer from Rluc to two neighboring acceptors.

The specificity of the observed interaction between Ste2p receptors was investigated by comparison to BRET measurement in cells expressing Rluc fused to the Na+/K+ ATPase Pma1p (48), a protein that is not known to undergo any interaction with Ste2p and that is one of the most abundant plasma membrane proteins in yeast. The level of energy transfer between Pma1p–Rluc and truncated Ste2p–GFP2 (Fig. 4A) was not significantly different from that of cells expressing Pma1p–Rluc alone (data not shown).

In contrast to previous studies that failed to detect FRET transfer between full-length Ste2p receptors, we detected significant energy transfer between full-length receptors fused to GFP2 and Rluc (Fig. 4B). After background subtraction, the BRET ratio for this interaction was ~1%. The ultimate level of sensitivity of BRET in this system appears to be limited by the intensity of light output from the Renilla luciferase and the expressed levels of tagged proteins. Because light emission by the luciferase reaction is transient, the only ways to increase total photons counted in order to enhance discrimination between true BRET signals and the low background levels of Rluc emission at 515 nm are to increase the concentration of tagged molecules in the sample (which ultimately reduces excitation and emission because of sample turbidity) or to conduct the BRET measurements on many replicate samples. The BRET ratios from preparations containing Rluc- and GFP-tagged full-length receptors are very reproducible from sample to sample and remain significantly greater ($p < 0.01$) than the ratios observed from preparations containing Ste2p–Rluc alone or the combination of Pma1p–Rluc and Ste2p–GFP2 (Fig. 4B). Furthermore, we find that changes in energy transfer caused by mutations and alterations in relative expression of Rluc- and GFP-tagged full-length receptors follow the same general trends as for truncated receptors (see below). Thus, the energy

![Image of Figure 2. Improving the detection of Rluc emission in yeast. A, bioluminescence emission at 390 nm (Rluc trace) for strain A2756 (STE2–Rluc w/STE2–GFP2) in untreated yeast cells after the addition of 5 μM DBC. B, bioluminescence emission monitored in strain A2756 pretreated with a cycle of freezing and thawing. C, bioluminescence emission in a membrane preparation of A2756 from a 10-fold larger volume of cell culture.](image)

![Image of Figure 3. Monitoring BRET2 in yeast. BRET2 ratios in freeze-thawed cells for control strains A2740, expressing covalently fused Rluc and GFP2, and A2752, expressing separate plasmids encoding Rluc and GFP2, each under control of the GAL1 promoter, and A2742, expressing Rluc alone under GAL control. Note that the BRET ratios shown have not been corrected for background BRET observed in cells expressing Rluc alone. Inset, immunoblot using anti-GFP antibody to detect covalently fused Rluc-GFP2 expressed in strain A2740 (lane 1) and unfused GFP2 co-expressed with Rluc in strain A2752 (lane 2) (note that due to the addition of the yeast MORF library tags (34), both constructs experience an approximate 20-kDa migration shift.)](image)
transfer that we detect between full-length receptors, although insufficient, provides strong evidence for oligomerization of full-length Ste2p.

High levels of expression of receptors can increase spontaneous, random collision between proteins or promote oligomerization via low affinity interactions (49). Because C-terminal-truncated Ste2p receptors are present in increased numbers at the cell surface relative to full-length receptors (26), we wished to examine the possibility that the greater observed BRET efficiency for truncated receptors could be due to such random or low affinity interactions. Thus, we varied receptor expression levels by expressing full-length and truncated receptors from single copy or multicopy plasmids. As shown in Fig. 5A, the BRET efficiencies were not significantly affected when expression levels of both the donor and acceptor were changed in parallel either for full-length or truncated receptors. However, for both the full-length and truncated receptors, BRET ratios were greater ($p < 0.05$) when the BRET donor was expressed from a CEN plasmid and acceptor was expressed from a multicopy plasmid than when donor and acceptor were expressed at equal levels either in multicopy or CEN plasmids. This result is expected, since increased abundance of acceptor relative to donor increases the likelihood that any given donor will co-oligomerize with acceptor, as has been shown in a number of BRET saturation assays (50–52). Substituting Pma1p as the donor protein in this situation yielded negligible energy transfer with either full-length or truncated receptors (Fig. 5A).

As an additional check of the specificity of the observed dimerization of Ste2p receptors, the ability of untagged receptors to compete for co-oligomerization with co-expressed $\text{GFP}^2$-tagged receptors was examined. Expression of un-tagged full-length Ste2p from the normal chromosomal locus reduced energy transfer between plasmid-encoded full-length receptors by 40% when $\text{STE2}$-$\text{Rluc}$ was expressed from a CEN plasmid and $\text{STE2}$-$\text{GFP}^2$ was expressed from a multicopy plasmid (Fig. 5B). Under similar conditions, expression of untagged full-length Ste2p reduced energy transfer between C-terminal-truncated receptors by 25% (Fig. 5B). The fact that only partial inhibition is observed can be explained by the presence of substoichiometric amounts of untagged compared with tagged receptors in these cells.

### TABLE 1

| Strain     | Strain description          | Plasmids                          | GFP$^2$-tagged protein | Rluc-tagged protein | Host      |
|------------|-----------------------------|-----------------------------------|------------------------|---------------------|-----------|
| A3091      | Truncated Ste2p$^a$ with epitope | pMD1134 + pMD1139                 | Truncated Ste2p$^a$    | Truncated Ste2p$^a$ | A3102     |
| A2753      | Truncated Ste2p$^a$          | pMD1154 + pMD1155                 | Truncated Ste2p$^a$    | Truncated Ste2p$^a$ | A232      |
| A2935      | $\text{CEN} +$ multicopy truncated Ste2p$^a$ | pMD1154 + pMD1199                 | Truncated Ste2p$^a$    | Truncated Ste2p$^a$ | A232      |
| A2998      | $\text{CEN} +$ multicopy truncated Ste2p$^a$ | pMD1224 + pMD1226                 | Truncated Ste2p$^a$ (G56A/G60A) | Truncated Ste2p$^a$ (G56A/G60A) | A232      |
| A2913      | Truncated Ste2p$^a$ (G56A/G60A) | pMD1224 + pMD1226                 | Truncated Ste2p$^a$ (G56A/G60A) | Truncated Ste2p$^a$ | A232      |
| A2914      | Truncated Ste2p$^a$ (G56L/G60L) | pMD1225 + pMD1227                 | Truncated Ste2p$^a$ (G56L/G60L) | Truncated Ste2p$^a$ (G56L/G60L) | A232      |
| A3285      | $\text{CEN} +$ multicopy truncated Ste2p$^a$ (G56A/G60A) | pMD1224 + pMD1381                 | Truncated Ste2p$^a$ (G56A/G60A) | CEN-Truncated Ste2p$^a$ (G56A/G60A) | A232      |
| A3286      | $\text{CEN} +$ multicopy truncated Ste2p$^a$ (G56L/G60L) | pMD1198 + pMD1199                 | CEN Truncated Ste2p$^a$ (G56L/G60L) | Truncated Ste2p$^a$ | A232      |
| A2820      | CEN-truncated Ste2p$^a$      | pMD1198 + pMD1199                 | CEN-TRuncated Ste2p$^a$ | Truncated Ste2p$^a$ | A232      |
| A2756      | Ste2p                        | pMD1167 + pMD1168                 | Ste2p                  | Ste2p               | A232      |
| A3136      | $\text{CEN} +$ Ste2p$^a$    | pMD1232 + pMD1242                 | CEN Ste2p              | CEN Ste2p           | A3102     |
| A3090      | Ste2p                        | pMD1167 + pMD1168                 | Ste2p                  | Ste2p               | A3102     |
| A3160      | $\text{CEN} +$ multicopy Ste2p | pMD1167 + pMD1232                 | Ste2p                  | CEN Ste2p           | A3102     |
| A3164      | $\text{CEN} +$ multicopy Ste2p | pMD1167 + pMD1232                 | Ste2p                  | CEN Ste2p           | A3087$^c$|
| A3161      | $\text{CEN} +$ Ste2p + multicopy Ste2p (Y266C) | pMD1244 + pMD1232                 | Ste2p (Y266C)          | Ste2p (Y266C)       | A3102     |
| A3142      | $\text{CEN} +$ Ste2p + multicopy Ste2p (G56L/G60L/Y266C) | pMD1263 + pMD1222 | Ste2p (G56L/G60L/Y266C) | Ste2p (G56L/G60L/Y266C) | A3102     |
| A3328      | $\text{CEN} +$ Ste2p + multicopy Ste2p (P40AS) | pMD1141 + pMD1232                 | Ste2p (P40AS)          | CEN Ste2p           | A3102     |
| A2740      | GFP$^2$-Rluc                  | pMD1134                           | Soluble fusion         | Soluble fusion      | A2638$^b$|
| A2742      | Rluc                         | pMD1147                           | NA$^a$                 | NA$^a$              | A2638$^b$|
| A2752      | Rluc + GFP$^2$                | pMD1147 + pMD1148                 | Soluble GFP$^2$        | Soluble GFP$^2$     | A2638$^b$|
| A3137      | GFP$^2$-Spel-Rluc             | pMD1301                           | Soluble fusion         | Soluble fusion      | A3087$^c$|
| A3138      | GFP$^2$-3HA-Rluc              | pMD1303                           | Soluble fusion         | Soluble fusion      | A3087$^c$|
| A3139      | GFP$^2$-6HA-Rluc              | pMD1304                           | Soluble fusion         | Soluble fusion      | A3087$^c$|
| A2847      | Pma1p with truncated Ste2p$^a$ | pMD1154 + pMD1209                 | Truncated Ste2p$^a$    | Pma1p               | A232      |
| A2848      | Pma1p with Ste2p              | pMD1167 + pMD1209                 | Ste2p$^a$              | Pma1p               | A232      |
| A3171      | Pma1p with Ste2p              | pMD1167 + pMD1209                 | Ste2p$^a$              | Pma1p               | A232      |
| A3092      | Internally fused Ste2p        | pMD1211 + pMD1212                 | Internally fused Ste2p | Internally fused Ste2p | A3102     |
| A3093      | Truncated Ste2p with an artificial tail | pMD1261 + pMD1262 | Truncated Ste2p with an artificial tail | Truncated Ste2p with an artificial tail | A3102     |
| A3094      | Internally fused Ste2p with an artificial tail | pMD1308 + pMD1266 | Internally fused Ste2p with an artificial tail | Internally fused Ste2p with an artificial tail | A3102     |
| A608       | $\text{ste}2\Delta$          | pMD228 + pMD107                    | NA$^a$                 | NA$^a$              | A232      |
| A2932      | Truncated Ste2p untagged      | pMD803 + pMD107                    | NA$^a$                 | NA$^a$              | A232      |

$^a$ Truncated Ste2p plasmids containing C252S.

$^b$ A230 is a MATa strain containing a chromosomal copy of the $\text{STE2}$ allele.

$^c$ GAL- inducible strains.

$^d$ NA, not applicable.
Because oligomerization of receptors does not appear to be affected by changes in receptor expression levels, the most likely remaining possible explanations for the large difference between the BRET ratios observed for full-length and truncated receptors are 1) a direct effect of the presence or absence of the C-terminal tail on the oligomeric state of the receptor or 2) an effect of an extra sequence between the transmembrane segments and the BRET donor and acceptor on the distance between the BRET reporters or on their relative orientations.

To look for specific effects of STE2 C-terminal tail sequences on receptor oligomerization, we inserted unrelated sequences in place of the C-terminal receptor tails between truncated receptors and BRET reporters (Fig. 1). These inserted sequences consisted of either a 44-amino acid segment consisting of a triple tandem repeat of the c-myc epitope or a 164-amino acid sequence containing His6 and IgG binding ZZ domains derived from the C-terminal tag used for the MORF yeast genomic library (34). Membrane preparations from cells expressing receptors with the 44-amino acid extension exhibited a BRET ratio of ~8% (Fig. 6A), somewhat lower than the 11% seen for similar preparations from cells expressing truncated receptors without the inserted sequences. Preparations from cells expressing receptors with the 164-amino acid extension exhibited a BRET ratio of ~3% (Fig. 6A). The simplest explanation of this result is that the presence of the extra sequence moves the BRET donor and acceptor farther apart, thereby decreasing energy transfer. The observed decreases in BRET brought about by sequences that would not be expected to affect the oligomeric state of the receptors suggest that the decreased energy transfer observed for full-length receptors is at least in part due to increased distance or altered relative orientation of the BRET proteins fused at different distances from the membrane. Fig. 6B shows that fusion of the BRET proteins to the C-terminus of truncated receptors does not impair the ability to signal, in agreement with previous reports (47).
The NH$_2$-to-COOH distance for each of the two Z domains in the 164-amino acid extension is about 50 Å (53).

The intrinsic dependence of energy transfer efficiency on the distance between the donor and acceptor in the BRET2 system was examined using a soluble fusion protein in which GFP$^2$ is covalently attached to \(\text{Rluc}\) with either three or six tandemly repeated copies of the influenza 12CA5 HA epitope tag cloned into its linker region. The BRET ratio from freeze-thawed cells expressing these constructs decreased as the length of sequence between them increased (Fig. 6C), but significant BRET was still detectable when the linker contained six copies of the epitope. Energy transfer for the shortest construct in this series was slightly higher than that observed for a construct of similar length but with a somewhat different linker sequence shown in Fig. 3. Expression levels of the constructs containing different length linkers were similar based on Western blotting against \(\text{Rluc}\) (Fig. 6D). The relatively modest (~2- and ~7-fold) decreases in BRET seen upon adding the longer linkers indicate that the distance between the chromophores in \(\text{Rluc}\) and GFP$^2$ is much greater than 20 Å even when they are connected by the shortest linker. The NH$_2$-to-COOH distance of a single copy of the 12CA5 epitope is ~20 Å in the crystal structure of influenza hemagglutinin (54). Given the strong \((1/r^6)\) distance dependence of energy transfer (55), the 7-fold decrease in energy transfer resulting from the insertion of six of these epitopes (in unknown orientations with respect to each other) must result from a relatively small increase on top of a large intrinsic separation. We expect that the linker sequences would allow sufficient flexibility such that changes in energy transfer efficiency due to changes in the relative orientation of chromophores are not important.

The characteristic Förster distance for the BRET$^2$ system must be large, given the ability to detect significant transfer between donor and acceptor combinations that must be separated by at least 100 Å. This distance includes as many as 6 repeats of a 20-Å epitope structure plus the intrinsic separation of the \(\text{Rluc}\) and GFP$^2$ chromophores that are part of proteins of ~38 and ~28 kDa, respectively. Based on the known structure
of GFP (56), the minimum distance from the chromophore to its surface is about 20 Å, and we assume the chromophore-to-surface distance in Rluc is similar.

To further examine the role of the C-terminal tail in receptor oligomerization, we created internal fusion constructs in which either Rluc or GFP² was attached after residue Lys-304 of truncated receptors, and the remainder of the C-terminal tail starting at residue Thr-305 was re-attached at the C-terminal of Rluc or GFP² (Fig. 1). We were surprised to find that the BRET ratio for these internally fused receptors was ~5% (Fig. 7A), significantly lower than for constructs in which the reporters were fused to truncated receptors without the re-attached tails. This demonstrates that anchoring of the BRET reporters immediately adjacent to the transmembrane regions of truncated receptors is not in itself sufficient for efficient BRET transfer. To determine whether the low BRET efficiency of this internal fusion construct might be caused by specific sequences in the C-terminal tail that prevent dimerization, we created a second internal fusion construct in which the BRET reporters were attached to truncated receptors and the exogenous C-terminal tag sequence from the MORF yeast genomic library (34) was attached at the C termini of the reporters. The BRET ratio for these ZZ-domain-containing internal fusion constructs was 8% (Fig. 7A), still somewhat less than the 11% efficiency determined for C-terminal-truncated receptors without re-attached tails. Re-attachment of either the STE2 tail or the MORF tag in these constructs did not reverse the hypersensitive signaling responses characteristic of C-terminal truncation of receptors (data not shown). Fig. 7B shows that all these fusion proteins were well expressed. Thus, attachment of tail sequences at the C termini of the BRET reporters appears to alter the spatial relationships of the BRET proteins, but the presence of the STE2 tail at this ectopic position does not restore the normal pheromone sensitivity of full-length receptors.

Glycine residues 56 and 60 in TM1 of the α-factor receptor have been implicated in a glycosphingolipid-like dimerization motif (57) that can be disrupted by mutation of these residues to either alanine or leucine (57). Substitutions of residues with larger side chains at these positions reduced the observed inter-receptor FRET and also impaired the cell-surface targeting of Ste2p (57). We used BRET to assess the ability of receptors with mutations at Gly-56 and Gly-60 to dimerize. Truncated receptors containing the G56A/G60A mutations displayed ~7% energy transfer (Fig. 8A), and those containing the G56L/G60L mutations showed a more severe loss of dimerization to 4% BRET transfer (Fig. 8A). However, as was the case for normal receptors, overexpression of the acceptor fusion protein containing glycine mutations resulted in increases in BRET efficiency (to 10% for the G56A/G60A mutant and to nearly 8% for the G56L/G60L mutant). Because both of these efficiencies are significantly above background, the glycine substitutions decreased but did not completely abolish dimerization of C-terminal-truncated Ste2p. Fig. 8B shows the ability of these C-terminal-truncated receptor mutants to initiate signaling in response to pheromone. Truncated receptors containing the glycine to alanine substitutions are partially functional, exhibiting slightly reduced pheromone sensitivity compared with truncated receptors containing the normal glycines at these positions. However, the more dramatically dimerization-defective glycine-to-leucine mutants are completely nonfunctional, in agreement with previous assays of pheromone-induced growth arrest (57). Similar results were obtained for full-length receptors with these mutations (data not shown).

Role of Dimerization in the Dominant Negative Effects of Mutant Receptors—Several previous screens have uncovered mutant receptor alleles that act in a dominant negative manner to attenuate signaling by co-expressed normal receptors (1, 25, 26). In addition, normal receptors co-expressed with constitu-
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FIGURE 8. Substitutions in the GXXXG motif inhibit dimerization. A, BRET in freeze-thawed cells for C-terminal-truncated Ste2p receptors that contain either G56A/G60A (CEN/multicopy, A3285; multicopy/multicopy, A2913) or G56L/G60L (CEN/multicopy, A3286; multicopy/multicopy, A2914) mutations in TM1. Rluc-tagged receptors were expressed either from CEN or multicopy plasmids, and GFP2-tagged receptors were expressed from multicopy plasmids. WT, wild type. B, FUS1-LacZ induction in response to α-factor binding for the following strains expressing BRET-tagged C-terminal-truncated receptors: no receptor (A608), C-terminal-truncated Ste2p (A2753), C-terminal-truncated Ste2p (G56A/G60A) (A2913), and C-terminal-truncated Ste2p (G56L/G60L) (A2914).

FIGURE 9. Heterodimerization of wild type and dominant negative mutant receptors. A, BRET transfer for membrane preparations from strains expressing full-length Ste2p-Rluc from CEN plasmids and overexpressing either Ste2p-GFP2 (A3160), Ste2p (F204S)-GFP2 (A3328), Ste2p (Y266C)-GFP2 (A3161), or Ste2p (G56S/G60A/Y266C)-GFP2 (A3142) from multicopy plasmids. Energy transfer for a strain (A3136) co-expressing Ste2p receptors from CEN plasmids is shown for comparison. WT, wild type. B, immunoblot of cell lysates from strains expressing CEN Ste2p-Rluc and multicopy or CEN Ste2p-GFP2 alleles (lane 1, Ste2p-Rluc alone; lanes 2–4, A3160; lanes 5–7, A3161; lanes 8–10, A3142; lanes 11–13, A3136) and probed with anti-GFP antibodies. Three isolates of each strain are represented.

atively active or hypersensitive receptors act in a dominant manner to suppress constitutive and hypersensitive signaling. There are two distinct classes of models to explain such dominant effects of receptors. The first is titration of G protein. If the amount of G protein in cells is limiting and if unactivated receptors are maintained in a complex with GDP-bound G protein, expression of one receptor allele in cells could prevent signaling via another co-expressed allele by preventing the other allele from gaining access to G protein. The second is receptor oligomerization. If interactions between co-oligomerized receptors are important for signaling, formation of oligomers containing defective receptors could inhibit signaling by normal receptors. Furthermore, co-oligomerization of normal receptors with dominant receptor alleles that are defective in intracellular trafficking could prevent normal receptors from reaching the plasma membrane.

To determine whether dominant negative effects of mutant receptors might be mediated by oligomerization, we assayed for BRET transfer between dominant negative and normal receptor alleles. Because C-terminal-truncated receptors do not exhibit dominant negative effects (28), this would not have been possible using previous FRET-based approaches, which are unable to detect oligomerization of full-length receptors. We find that Ste2p receptors containing the substitutions Y266C and F204S, two of the strongest known dominant negative alleles of STE2, can co-oligomerize with normal receptors (Fig. 9A). Such co-oligomerization is observed in membranes from cells expressing dominant negative receptors from a multicopy plasmid and normal receptors from a CEN plasmid, the same combinations of receptors that lead to dominant negative effects (25, 26). The BRET efficiency between either the Y266C or F204S mutant with normal receptors was comparable with that observed between two wild-type receptors (Fig. 9A).

To further test the role of receptor oligomerization in the dominant actions of receptors, we examined whether mutations that reduce the extent of oligomerization could also block the dominant negative behaviors of mutant receptors. To accomplish this, the G56L and G60L mutations were introduced into receptors that also contain the dominant negative Y266C mutation, and these receptors were expressed in cells that also co-express normal receptors. This would be expected to at least partially disrupt the formation of oligomers involving the dominant negative mutations, thereby increasing the formation of homo-oligomers of normal receptors. As seen in Fig. 9A, an allele containing the triple G56L/G60L/Y266C substitutions exhibited a 4-fold reduction in energy transfer from normal to mutant receptors compared with a strain expressing the same alleles without the glycine substitutions, indicative of
severely impaired abilities of the dominant negative receptors to co-oligomerize with the normal receptors. Immunoblotting of whole-cell extracts shown in Fig. 9B demonstrates that the reduction in the efficiency of BRET transfer to the G56L/G60L-containing dominant negative receptors is not the result of a decrease in receptor expression, as levels of GFP-tagged receptors for wild-type, Y266C, and G56L/G60L/Y266C alleles are all comparable, and the levels of these receptors expressed from multicopy plasmids are much greater than the levels of normal receptors expressed from CEN plasmids.

### TABLE 2

| Strain | Strain description | Plasmid | Host |
|--------|--------------------|---------|------|
| A338   | Ste2               | pMD240  | A230 |
| A430   | Vector             | pMD228  | A230 |
| A3066  | Ste2p (G56A/G60A)  | pMD1217 | A232 |
| A3067  | Ste2p (G56L/G60L)  | pMD1218 | A232 |
| A3214  | CEN Ste2p (Y266C)  | pMD167  | A230 |
| A478   | Ste2p (Y266C)      | pMD294  | A230 |
| A3062  | Ste2p (G56A/G60A/Y266C) | pMD1287 | A230 |
| A3064  | Ste2p (G56L/G60L/Y266C) | pMD1288 | A230 |
| A479   | Ste2p (Y266C)      | pMD294  | A232 |
| A3063  | Ste2p (G56A/G60A/Y266C) | pMD1287 | A232 |
| A3065  | Ste2p (G56L/G60L/Y266C) | pMD1288 | A230 |
| A3293  | Ste2p (F204S)      | pMD1205 | A230 |
| A3319  | Ste2p (G56A/G60A/F204S) | pMD1409 | A230 |
| A3320  | Ste2p (G56L/G60L/F204S) | pMD1410 | A230 |
| A3325  | Ste2p (F204S)      | pMD1205 | A232 |
| A3326  | Ste2p (G56A/G60A/F204S) | pMD1409 | A232 |
| A3327  | Ste2p (G56L/G60L/F204S) | pMD1410 | A232 |

The role of receptor co-oligomerization in the dominant negative actions of receptors was tested by comparing dominant signaling behavior of the Y266C receptor allele with the behavior of the triple mutants alleles G56A/G60A/Y266C and G56L/G60L/Y266C (Table 2). As reported previously (25), co-expression of the Y266C mutant with normal receptors severely inhibits the FUS1-lacZ response to α-factor compared with cells co-expressing normal receptors (Fig. 10A). In contrast, strains co-expressing the G56A/G60A/Y266C mutant with normal receptors exhibit less inhibition of the FUS1-lacZ response, and cells expressing the G56L/G60L/Y266C exhibit no inhibition of the FUS1-lacZ response compared with cells expressing only normal receptors (Fig. 10A). In the case of the G56A/G60A/Y266C allele, these effects are not likely to be due to any reduction in levels of expression of the triple mutant receptors, since Fig. 10C shows that the cellular levels of the triple mutant containing the G56A/G60A substitutions are comparable with those of the receptor containing Y266C alone. In the case of the G56L/G60L/Y266C allele, cellular levels of the mutant receptor were in fact reduced compared with the allele containing Y266C alone, so that the loss of dominant negative effects may result at least in part from lower expression. The substitutions at Gly-56 and Gly-60 had similar effects on dominant negative receptors containing the F204S mutation (Fig. 10B); combination of G56A/G60A with F204S inhibited the dominant negative effects of F204S to a lesser degree than the combination with G56L/G60L. The assays of the pher-
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**TABLE 3**

| Ste2p plasmid allele | Chromosomal \( \text{STE2} \) allele | \( B_{\text{max}} \) | \( K_d \) |
|----------------------|------------------------------------|-----------------|--------|
| Multicopy \( \text{Ste2p} \) | \( \text{STE2} \) | 13.7 ± 1.8 | 3.0 ± 0.4 |
| Empty vector | \( \text{STE2} \) | 1.6 ± 0.03 | 3.5 ± 0.4 |
| Multicopy \( \text{Ste2p} (\text{G56A}/\text{G60A}) \) | \( \text{ste2} \Delta \) | 2.3 ± 0.3 | 14.3 ± 4.7 |
| Multicopy \( \text{Ste2p} (\text{G56L}/\text{G60L}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{Y266C}) \) | \( \text{STE2} \) | 3.0 ± 0.1 | 43.4 ± 6.8 |
| Multicopy \( \text{Ste2p} (\text{G56A}/\text{G60A}/\text{Y266C}) \) | \( \text{ste2} \Delta \) | 3.3 ± 0.6 | 26.5 ± 6.3 |
| Multicopy \( \text{Ste2p} (\text{G56L}/\text{G60L}/\text{Y266C}) \) | \( \text{ste2} \Delta \) | 1.7 ± 0.1 | 6.4 ± 2.3 |
| Multicopy \( \text{Ste2p} (\text{Y266C}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{G56A}/\text{G60A}/\text{Y266C}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{G56L}/\text{G60L}/\text{Y266C}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{F204S}) \) | \( \text{STE2} \) | 2.0 ± 0.1 | 4.8 ± 0.5 |
| Multicopy \( \text{Ste2p} (\text{G56A}/\text{G60A}/\text{F204S}) \) | \( \text{STE2} \) | 2.1 ± 0.1 | 5.7 ± 0.3 |
| Multicopy \( \text{Ste2p} (\text{G56L}/\text{G60L}/\text{F204S}) \) | \( \text{STE2} \) | 1.3 ± 0.1 | 4.2 ± 0.6 |
| Multicopy \( \text{Ste2p} (\text{F204S}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{G56A}/\text{G60A}/\text{F204S}) \) | \( \text{ste2} \Delta \) | ND | ND |
| Multicopy \( \text{Ste2p} (\text{G56L}/\text{G60L}/\text{F204S}) \) | \( \text{ste2} \Delta \) | ND | ND |

omone response shown in Figs. 10, A and B, were performed with receptors that were not fused to BRET reporters. Similar effects were observed with receptor alleles containing BRET reporters (data not shown; however, the overall magnitude of the dominant negative effects of the Y266C mutation were reduced in strains containing the BRET reporter-fused receptors, consistent with previous reports that alterations at the C-terminal of the receptor can affect dominant negative behavior (25)).

We also examined the binding of ligand to intact cells expressing dominant negative receptors (Table 3). In our host strain containing a deletion of the chromosomal \( \text{STE2} \) gene, no ligand binding to dominant negative Y266C or F204S mutant receptors at the cell surface could be detected when these alleles were expressed from multicopy plasmids. Because the overall cellular levels of these mutant receptors detected by immunoblotting are comparable with the levels of normal receptors expressed from multicopy plasmids and are well above the cellular levels of chromosomally-encoded Ste2p (Fig. 10C and Ref. 26), the lack of binding to these cells indicates either that most of the mutant receptors are sequestered inside cells or that these alleles are defective for ligand binding. However, the low but significant levels of \( \alpha \)-factor responsiveness by cells expressing these mutant receptors indicates that some small number of receptors in fact reach the cell surface and are capable of initiating a pheromone response when provided with adequate concentrations of pheromone. A predominant intracellular location of GFP\(^2\) fused to dominant negative receptors was detected using fluorescence microscopy (results not shown). However, significant intracellular localization of GFP\(^2\) fused to normal receptors expressed from multicopy plasmids was also detected, making it difficult to draw firm conclusions about differences in cell surface expression based on microscopy, especially in view of the efficient signaling that can be mediated by very low levels of receptors at the cell surface (58).

When Y266C or F204S mutant receptors were expressed from multicopy plasmids introduced into cells containing a chromosomal copy of a normal \( \text{STE2}^+ \) allele, the number of binding sites detected was slightly higher than that observed when the chromosomal \( \text{STE2}^+ \) is expressed alone (Table 3). In the case of the Y266C allele, the ligand binding affinity of sites on these cells was significantly weaker than that of normal receptors. This reduction in affinity in combination with the lack of any detectable binding to cells expressing the Y266C allele alone indicates that the sites on cells co-expressing normal and mutant receptors are composed of hetero-oligomers. Apparently, the presence of normal receptors in the oligomers facilitates transport of the mutant receptors to the cell surface. The observed reduction in affinity compared with normal receptors can be explained in two ways; 1) hetero-oligomerization with mutant receptors may reduce the ligand binding affinity of the normal receptors, or 2) the binding assay may detect an average affinity of normal receptors (with normal binding affinity) and mutant receptors (with reduced binding affinity) that reach the surface as hetero-oligomers. In either case, because cells expressing these hetero-oligomers exhibit severely impaired responses to \( \alpha \)-factor, the hetero-oligomers of normal and mutant receptors are defective for signaling function even though they are targeted to the cell surface.

Cells co-expressing normal and F204S mutant receptors display cell-surface binding sites with near-normal ligand affinities (Table 3 and Ref. 26) even though they are severely defective in pheromone signaling. If, as suggested by previous results (26), the F204S receptor allele is capable of being transported to the cell surface but is defective for ligand binding, then the observed binding sites with normal affinity must arise from the presence of normal receptors that reach the cell surface as co-oligomers with the mutant receptors. On the other hand, if the F204S mutants are in fact capable of binding ligand with nearly normal affinity but are defective for transport to the cell surface when expressed alone, then the observed binding sites would be composed of both normal and mutant receptors that can reach the cell surface as signaling-defective co-oligomers.

Introduction of the G56L/G60L substitutions into an otherwise normal receptor results in a complete loss of binding sites at the cell surface (Table 3 and Ref. 57). Introduction of the weaker G56A/G60A substitutions leads to expression of a low affinity of normal receptor results in a complete loss of binding sites at the cell surface (Table 3 and Ref. 57). Introduction of the weaker G56A/G60A substitutions leads to expression of a low affinity of normal receptors (with normal binding affinity) and mutant receptors (with reduced binding affinity) that reach the surface as hetero-oligomers. In either case, because cells expressing these hetero-oligomers exhibit severely impaired responses to \( \alpha \)-factor, the hetero-oligomers of normal and mutant receptors are defective for signaling function even though they are targeted to the cell surface.
that contain either the Y266C or F204S mutation in cis with
mutations at Gly-56 and Gly-60 are not detectably expressed at
the cell surface, as expected, since this is a combination of two
classes of mutations that each decrease cell-surface expression
of receptors.

Cells co-expressing the G56L/G60L/Y266C mutants with
a normal chromosomally encoded STE2 \( {^+} \) express the same
number of binding sites that would be expected for the chro-
mosomal STE2 \( {^+} \) alone, with the same affinity as the normal
allele. This indicates that the lack of dominant negative
behavior of this allele is due to its failure to have any effect on
normal receptor expression either because of inability to
co-oligomerize with normal receptors or because of
low level expression.

In contrast to the case for the G56A/G60A/Y266C allele, co-
expression of G56A/G60A/Y266C receptors with normal chro-
mosomally encoded STE2 leads to appearance of cell-surface
ligand binding sites that are more abundant than those
observed on cells expressing the chromosomal STE2 \( {^+} \) alone.
This means that some of the mutant receptors appear on the
cell surface. Because no cell-surface binding could be detected
in cells expressing the G56A/G60A/Y266C allele by itself, this
must result from some co-oligomerization with normal recep-
tors, consistent with detection of residual BRET transfer (Fig.
8A) and with retention of partial dominant negative behavior
(Fig. 10A) by G56A/G60A-containing alleles. The affinity of
ligand binding to sites on the surface of cells co-expressing
mutant G56A/G60A/Y266C and normal receptors is lower
than the affinity for normal receptors expressed alone.
Although we were not able to determine a binding affinity for
the G56A/G60A/Y266C allele alone, both the Y266C and the
G56A/G60A substitutions resulted in lower than normal bind-
ing affinities (Table 3). Thus, it is likely that at least some of
the sites appearing at the cell surface are composed of hetero-oli-
gomers of normal and mutant receptors that bind ligand with
reduced affinity either because of a heterogeneous population
of binding sites in the oligomer or because of cooperative inter-
actions between co-oligomerized normal and mutant recep-
tors. Cells co-expressing normal and G56A/G60A/F204S
mutant receptors display ligand binding sites with approxi-
mately the same affinity as normal receptor. These sites are
either composed of 1) homo-oligomers of normal receptors
that escape from co-oligomerization with the mutants because
of the partial defect in oligomerization resulting from the
G56A/G60A substitution or 2) hetero-oligomers of normal and
mutant receptors that exhibit near-normal binding affinities
(see above).

**DISCUSSION**

Although there is evidence that many different GPCRs oli-
gomerize, the functional effects of such oligomerization remain
poorly understood in most cases (2, 3, 42, 59). In the yeast pher-
omone response pathway, oligomerization of receptors has
been proposed as a possible explanation of dominant effects of
certain receptor alleles. However, this has been difficult to con-
firm. A previous application of FRET for the study of oligomer-
ization of α-factor receptors was only capable of detecting
interactions between truncated receptors lacking the C-termi-
nal tail, whereas the dominant effects of receptors are only
observed for full-length alleles (28). We demonstrate here that
the low background and wide spectral separation between
donor and acceptor emission of the BRET\(^2\) system make it pos-
sible to achieve the sensitivity necessary to detect oligomeriza-
tion of both full-length and truncated α-factor receptors in
yeast cells. The ability of BRET to detect oligomerization of
full-length receptors allows direct demonstration of co-oli-
gomerization of normal and dominant negative receptor alleles.
Furthermore, the detection of BRET transfer between full-
length receptors together with the insensitivity of the efficiency
of energy transfer to levels of receptor expression demonstrate
that receptor oligomerization is a normal phenomenon in yeast
cells, not an abnormal condition induced by the elevated levels
of accumulation of truncated receptors in yeast plasma mem-
branes. The insensitivity of BRET to expression levels also
implies that the equilibrium constant for homo-oligomeriza-
tion of receptors is favorable enough to drive oligomerization
over a range of receptor abundances.

Energy transfer between full-length receptors was much less
efficient than between receptors with truncated C-terminal
tails. Because BRET transfer between full-length receptors is
altered by the same perturbations that affect truncated recep-
tors such as mutations of putative oligomerization contacts and
competition from co-expressed untagged receptors, the reduced
BRET transfer between full-length receptors appears to
result from a greater distance of separation between BRET
reporters attached to the ends of C-terminal tails rather than
from differences in the extent of oligomerization between full-
length and truncated receptors. Based on the ~10-fold differ-
ce in BRET efficiency between full-length and truncated
receptors, on an estimate that the closest separation between
chromophores of the BRET reporters fused to truncated recep-
tors is 50 Å (calculated from the known and estimated dimen-
sions of GFP and Rluc, respectively, see above), and on the 1/\( r^6 \)
distance dependence of energy transfer, the increase in distance
between the donor and acceptor in comparing truncated to
full-length receptors is about 23 Å. Decreases in BRET effi-
ciency were also observed when sequences unrelated to STE2
were inserted between the transmembrane region of the recep-
tor and the BRET reporters.

The length of amino acid sequence connecting transmem-
brane segments of the receptor to BRET reporters is not the
only factor affecting BRET efficiency of different full-length
and truncated receptor constructs. We observed a decrease in the
efficiency of BRET transfer between Rluc and GFP fused to
C-terminal-truncated receptors when extra sequences were at
the C termini of the BRET reporters (see Fig. 1). This decrease
occurred whether the extra sequences were derived from the
C-terminal tail of Ste2p or from an unrelated source. This
means that attachment of the BRET reporters directly adjacent
to transmembrane regions of Ste2p is not sufficient to guaran-
tee close proximity of donor and acceptor. The appended
sequences are apparently interposed into the interacting sur-
face between the Rluc and GFP of oligomerized receptors,
increasing their separation (compare models A and Cin Fig. 11).
Re-attachment of the normal C-terminal sequences at this site

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FIGURE 11. Models of the relative dispositions of the RLuc donor and GFP² acceptor compatible with observed BRET efficiencies for α-factor receptors with truncated C-terminal tails (A), full-length receptors (B), and receptors containing reporters fused internally just C-terminal to the seventh transmembrane segment (C).

does not allow them to play their normal roles in down-regulation of receptor signaling.

Mutation of the GXXXG motif in the first transmembrane segment of Ste2p has previously been reported to interfere with dimerization of truncated receptors (57), and we report here that substitution of more bulky residues for glycine at Gly-56 and Gly-60 of Ste2p reduces BRET efficiency between receptors. However, the sensitivity of the BRET system made it possible to determine that the introduction of alanine or leucine at these positions was not sufficient to completely abolish dimerization, consistent with recent reports that sequences outside these positions can interfere with normal signaling.

An unresolved general question in considering mechanisms of GPCR activation is whether there are functional interactions among co-oligomerized receptors such that signaling by an oligomer requires cooperation between the constituent receptors. The enhanced sensitivity of the BRET technique allows detection of co-oligomerization of full-length normal receptors with co-expressed full-length dominant negative mutant receptors, conditions under which the dominant negative effects of the mutant receptors are observed. Furthermore, we find that cells that co-express normal receptors and dominant negative receptors with the Y266C mutation accumulate slightly higher levels of ligand binding sites at the cell surface than cells that just express a normal chromosomal copy of STE2. Because these surface-expressed proteins do not signal efficiently and because they bind ligand with lower affinity than normal receptors, they cannot be oligomers of normal receptors (the presence of only a very low number of normal cell-surface receptors is sufficient for eliciting such a response (58)). They also cannot be homo-oligomers of the Y266C mutant receptors since the mutant receptors when expressed alone are not efficiently accumulated at the cell surface. Thus, the ligand binding sites in these cells appear to be composed of hetero-oligomers of normal and mutant receptors. The inability of such hetero-oligomers to signal despite the fact that they contain normal receptor monomers and are present at the cell surface leads to the conclusion that functional cooperation between the components of these GPCR oligomers is required for efficient signaling.

A related scenario can be invoked to describe hetero-oligomers of normal receptors with mutant receptors containing another dominant negative allele with the substitution F204S. In this case, co-expression of normal and mutant receptors leads to appearance of cell-surface sites with normal binding affinity. The sites are not homo-oligomers of normal receptors, since they fail to elicit a normalpheromone response, and they are also not homo-oligomers of mutant receptors, since no such sites can be detected in cells expressing the F204S allele alone. Instead, they appear to be composed of hetero-oligomers that are defective in pheromone-dependent signaling.

The defect in signaling exhibited by normal receptors that are constituents of hetero-oligomers of normal and dominant negative mutant receptors could occur at the level of ligand binding, at the level of receptor activation, or at the level of interaction with G protein. Such cooperation is not likely to be a special property of the particular dominant negative alleles used in this study, since dominant negative alleles are common among loss-of-function mutations in the STE2 gene (25, 26). The ease of isolating such mutations implies that the dominant effects are due to the loss of some receptor function and not to some special gain-of-function that allows the mutant to interfere with normal signaling.

The role of oligomerization in mediating the dominant negative effects of receptors is supported by the loss of dominant negative effects that occurred upon combination of substitutions in the putative GXXXG dimerization motif in cis with the Y266C or F204S dominant negative mutations. Although this could in principle be the result of reduced expression of the mutant alleles, we find that mutant receptors containing the G56A/G60A substitutions in combination with Y266C are present in cell extracts at approximately normal levels. The sensitivity of the BRET assay we have employed was sufficient to demonstrate that the G56A/G60A substitutions result in only a partial defect in oligomerization that provides only partial alleviation of dominant negative effects.

Oligomerization of receptors is one of two major models that have previously been proposed for explaining dominant negative effects of mutant receptors (1, 25, 28). An alternative explanation for dominant negative effects is the sequestration of limiting numbers of G proteins into inactive receptor-G protein complexes. Evidence for such sequestration has been based on indirect genetic experiments such as partial reversal of dominant negative effects by overexpression of G protein subunits. However, since an imbalance between the number of α subunits compared with the number of βγ subunits can lead to either enhancement or inhibition of signaling, it is difficult to be certain that G protein overexpression directly overcomes the effects of G protein sequestration. Furthermore, we do not find that reductions in the abundance of normal and mutant receptors compared with G protein subunits lead to the loss of dominant negative effects that would be expected based on the
model involving G protein sequestration. However, the present study does not rule out the possibility that inactive receptors may exist in a pre-coupled complex with G proteins and that the effects of some dominant negative alleles may be explained by this mechanism.

Oligomerization of receptors has previously been reported to occur at early stages of the secretory pathway (5, 51, 57, 59) and may be required for efficient transit of the pathway (5, 6, 59). Three of the mutations we have studied, the dominant negative Y266C and F204S alleles and the partially oligomerization defective G56A/G60A allele, are present at normal levels in cell extracts but at undetectable levels at the cell surface. This could reflect either an inherent defect in ligand binding by these receptors or an inability to reach the cell surface. Because it has previously been reported that the Y266C allele binds ligand with slightly reduced affinity (26) and because we can detect cell-surface binding to C-terminal-truncated receptors with this substitution (data not shown), it is likely that for this allele at least the observed oligomerization must primarily reflect the behavior of receptors that are present in internal cellular membranes. The loss of cell surface expression caused by mutations in the GAXXG motif does not necessarily establish any role for oligomerization in trafficking to the cell surface, since mutations that do not diminish oligomerization (such as Y266C and F204S) also lead to severe reduction in the abundance of receptors at the cell surface and since the mutations at Gly-56 and Gly-60 may have direct effects on receptor function or trafficking in addition to their effects on oligomerization. Dosil et al. (26) found that co-expression of dominant negative receptors with normal receptors did not alter the sub-cellular distribution of normal receptors, consistent with our finding that hetero-oligomers of normal and mutant receptors are expressed at the cell surface.

Because truncated receptors lacking C-terminal tails can effectively oligomerize, the reported failure of C-terminal-truncated α-factor receptors to exhibit dominant negative effects on signaling (28) remains unclear. Possible explanations include 1) lower overall cellular levels of truncated receptors, leading to a reduction in the efficiency of hetero-oligomer formation (despite the increased abundance of truncated receptors at the cell membrane), 2) a role for the tail in receptor cooperativity such that hetero-oligomerization of a defective truncated receptor with a normal full-length receptor does not inhibit the function of the normal receptor, and 3) partial compensation for defective receptor alleles by the hypersensitivity, lack of desensitization, and increased cell surface targeting of truncated receptors.

The results presented here demonstrate that there is oligomerization of full-length α-factor receptors present at normal abundance in yeast membranes, that such oligomerization provides an explanation for the effects of dominant negative receptor mutations, and that active cooperation between co-oligomerized receptors is required for effective signaling by receptors present at the cell surface. Further studies will address the mechanisms of the observed cooperative effects on signaling.

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