G-protein-coupled receptors function as oligomers *in vivo*

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Hormones, sensory stimuli, neurotransmitters and chemokines signal by activating G-protein-coupled receptors (GPCRs) [1]. Although GPCRs are thought to function as monomers, they can form SDS-resistant dimers, and coexpression of two non-functional or related GPCRs can result in rescue of activity or modification of function [2–10]. Furthermore, dimerization of peptides corresponding to the third cytoplasmic loops of GPCRs increases their potency as activators of G proteins *in vitro* [11], and peptide inhibitors of dimerization diminish β_2-adrenergic receptor signaling [3]. Nevertheless, it is not known whether GPCRs exist as monomers or oligomers in intact cells and membranes, whether agonist binding regulates monomer–oligomer equilibrium, or whether oligomerization governs GPCR function. Here, we report that the α-factor receptor, a GPCR that is the product of the *STE2* gene in the yeast *Saccharomyces cerevisiae*, is oligomeric in intact cells and membranes. Coexpression of receptors tagged with the cyan or yellow fluorescent proteins (CFP or YFP) resulted in efficient fluorescence resonance energy transfer (FRET) due to stable association rather than collisional interaction. Monomer–oligomer equilibrium was unaffected by binding of agonist, antagonist, or G protein heterotrimers. Oligomerization was further demonstrated by rescuing endocytosis-defective receptors with coexpressed wild-type receptors. Dominant-interfering receptor mutants inhibited signaling by interacting with wild-type receptors rather than by sequestering G protein heterotrimers. We suggest that oligomerization is likely to govern GPCR signaling and regulation.

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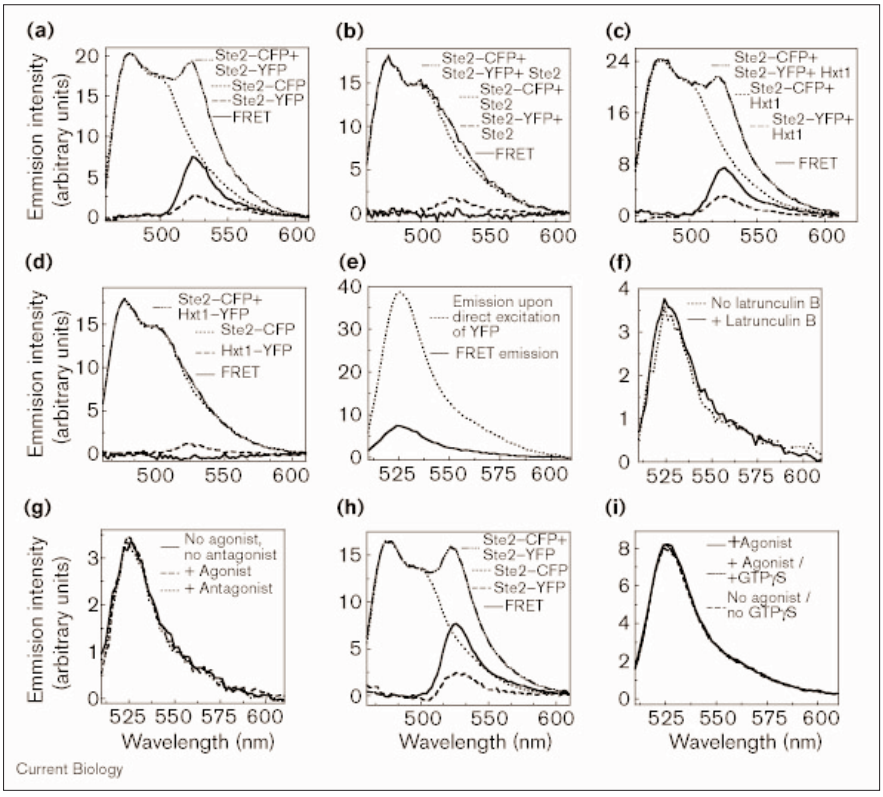
Results and discussion

To determine whether the α-factor receptor is an oligomer *in vivo*, we performed FRET experiments in conjunction with cell biological and genetic studies. FRET experiments were performed using cells expressing wild-type levels of truncated α-factor receptors lacking their cytoplasmic carboxy-terminal regulatory domains (Ste2Δtail–CFP, donor; Ste2Δtail–YFP, acceptor). Tagging truncated receptors with the green fluorescent protein (GFP) does not alter the cell-surface expression, agonist-binding affinity, signaling efficiency or internalization defect of the mutant receptor [12]. FRET was detected between Ste2Δtail–CFP and Ste2Δtail–YFP coexpressed at wild-type levels (Figure 1a). In contrast, FRET was not detected between CFP- and YFP-tagged full-length receptors (data not shown), indicating a dependence on interfluorophore distance, orientation or mobility.

Several results indicated that FRET was due to a direct, specific, homophilic interaction between tailless receptors, and not to dimerization through CFP and YFP, non-specific collisional interaction, or an indirect interaction mediated by the cortical actin cytoskeleton. First, FRET was not detected when untagged receptors were overexpressed (Figure 1b), even though the tagged receptors were expressed normally at the cell surface as indicated by quantitation of CFP and YFP fluorescence and by fluorescence microscopy (data not shown). Inhibition of FRET by untagged receptors was specific because overexpression of a plasma membrane glucose transporter (Hxt1) at a similar level did not affect FRET between tagged receptors (Figure 1c). Specific inhibition of FRET by overexpressed wild-type receptors also suggested that wild-type receptors oligomerize. Second, FRET was not detected between CFP-tagged tailless receptors and YFP-tagged glucose transporters (Figure 1d), indicating that random collisional interactions between plasma membrane proteins does not cause FRET. Third, a stable, specific interaction between α-factor receptors was indicated by the high apparent efficiency of FRET ($18 \pm 1\%$, $n = 7$; Figure 1e). Fourth, FRET was detected when the actin cytoskeleton was disassembled by treating cells with latrunculin B (Figure 1f).

The apparent efficiencies of FRET observed with control cells and cells treated with agonist or antagonist were indistinguishable ($18\% \pm 1$, $n = 6$), as indicated by dose-response and time-course experiments using sub-saturating to saturating ligand concentrations (Figure 1g and data not shown). Therefore, stabilization of α-factor receptors in either an active or inactive conformation was not accompanied by substantial changes in monomer–oligomer equilibrium. Energy transfer experiments using human β-adrenergic receptors have yielded similar results (S. Angers, A. Salahpour, E. Joly, S. Hilairet, D. Chelsky, M. Dennis and M. Bouvier, personal communication). Agonist or antagonist binding could, however, affect receptor distribution among

Figure 1



Use of FRET to detect oligomerization of α-factor receptors *in vivo*. Yeast cells expressed tailless α-factor receptors fused to CFP or YFP (Ste2–CFP or Ste2–YFP, referred to as Ste2Δtail–CFP and Ste2Δtail–YFP in the main text) and/or glucose transporters fused to YFP (Hxt1–YFP), as indicated. Untagged α-factor receptors (Ste2) or glucose transporters (Hxt1) were overexpressed where indicated. **(a–g)** Intact cells or **(h,i)** plasma membrane fractions were excited at the λ_{max} of CFP (440 nm), and fluorescence emission detected by scanning fluorometry. Fluorescence emission due to FRET was determined by subtracting the emission spectrum of cells expressing the CFP fusion alone, and the emission spectrum of cells expressing YFP fusion alone, from the emission spectrum of cells coexpressing CFP and YFP fusion proteins. In (e), the efficiency of FRET was determined by dividing the integrated FRET curve by the integrated emission curve obtained upon direct excitation of YFP at 490 nm. In (f,g,i), only the FRET emission curves are shown for experiments performed under the indicated conditions. All results shown here are representative of at least six independent experiments.

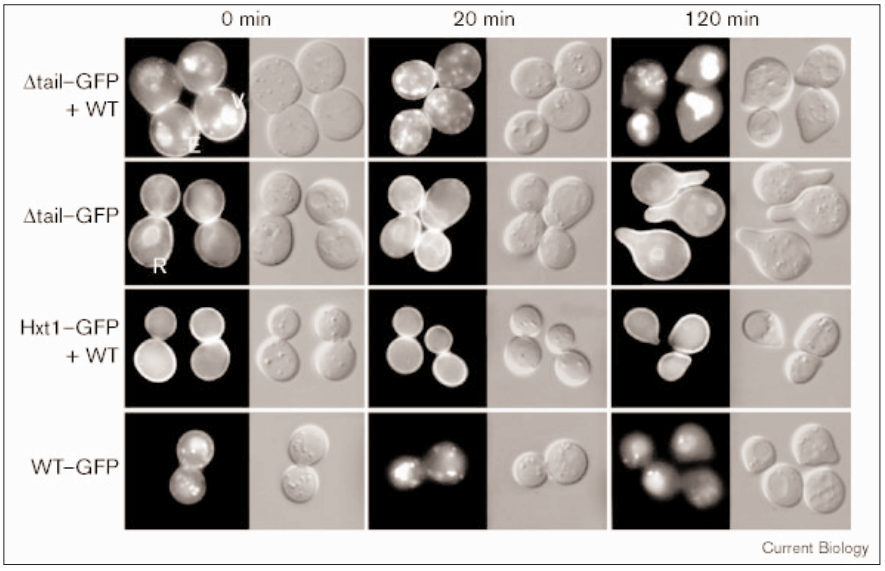
oligomeric states (for example, dimers and tetramers), which would not necessarily affect FRET efficiency.

FRET was also observed in experiments using purified plasma membrane fractions derived from cells coexpressing Ste2Δtail-CFP and Ste2Δtail-YFP (Figure 1h). This result allowed us to determine whether monomer–oligomer equilibrium is affected by the formation of receptor–G protein ternary complexes, or by the sustained activation and dissociation of G protein subunits from the receptor. The apparent efficiency of FRET ($20\% \pm 2\%$, $n = 11$) in plasma membrane fractions was indistinguishable whether receptors and G proteins were inactive (no agonist or GTPγS; Figure 1i), had formed ternary complexes (5 μM agonist without GTPγS; Figure 1i), or were activated and uncoupled (5 μM agonist and 50 μM GTPγS; Figure 1i). These results reinforced the conclusion that monomer–oligomer equilibrium is unaffected during signaling.

The endocytosis defect of GFP-tagged tailless receptors could be corrected by coexpressing untagged wild-type receptors (Figure 2), further demonstrating that α-factor receptor oligomerization occurs *in vivo*. In these cells, agonist-independent endocytosis of GFP-tagged tailless receptors was indicated by fluorescence labeling of endosomal vesicles and the large lysosome-like vacuole, as occurred when cells expressed only full-length GFP-tagged receptors (Figure 2). Cells coexpressing untagged wild-type receptors and GFP-tagged tailless receptors also displayed evidence of agonist-induced receptor internalization, as indicated by time-dependent decreases in the level of cell-surface GFP-tagged tailless receptors and increases in the levels of GFP fluorescence associated with endosomes and the vacuole (Figure 2). The ability of wild-type receptors to rescue the endocytosis defect of GFP-tagged tailless receptors was not due to stimulation of bulk internalization of plasma membrane lipids and proteins, because the Hxt1–GFP fusion was not internalized when coexpressed with untagged wild-type receptors either with or without agonist stimulation (Figure 2). In contrast, tailless GFP-tagged receptors expressed alone did not undergo basal or agonist induced endocytosis, as indicated by their persistence at the plasma membrane and their inability to localize to endosomes or the vacuole (Figure 2). Thus, α-factor receptors appear to be oligomeric in the absence or presence of agonist, in accord with results of FRET experiments.

To address whether oligomerization is important for receptor signaling, we identified and characterized a dominant-interfering mutant of the α-factor receptor. This mutant was termed M250I because of the amino-acid substitution it contained in the transmembrane segment VI. In cells

Figure 2

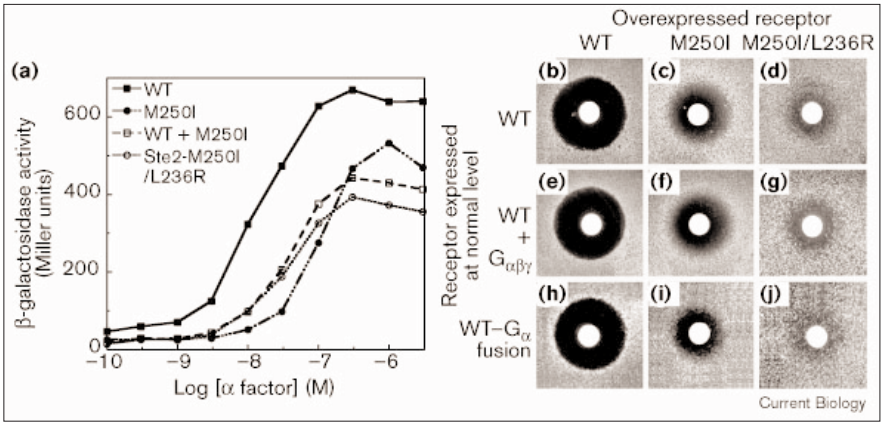


Oligomerization of α-factor receptors during endocytosis. Fluorescence microscopy was used to localize GFP-tagged tailless receptors coexpressed with untagged wild-type receptors (Δtail–GFP + WT) or alone (Δtail–GFP), GFP-tagged glucose transporters expressed with untagged wild-type receptors (Hxt1–GFP + WT), and wild-type receptors tagged with GFP (WT–GFP). Images were acquired before (0 min) or at the indicated times after the addition of agonist (α-factor, 5 μM). Endosomal vesicles (E), the lysosome-like vacuole (V) and the endoplasmic reticulum (R) are indicated, as documented previously [14].

expressing only the mutant receptor from a single-copy plasmid and its normal promoter on a single-copy plasmid, agonist-binding affinity was impaired ~10-fold (Kd = 19 nM versus 2 nM; data not shown) whereas cell-surface expression was reduced only slightly (4,600 sites per cell versus 6,000–8,000 sites per cell; data not shown). Furthermore, in these cells agonist potency was impaired nearly 10-fold (that is, EC_{50} was higher) relative to wild-type receptor controls, as indicated by dose-response curves for expression of an agonist-inducible reporter gene (*FUS1–lacZ*; Figure 3a).

When overexpressed, the dominant-interfering receptor could inhibit signaling by interacting with wild-type receptors, or by sequestering a limiting pool of G protein heterotrimers. The receptor interaction mechanism of inhibition was supported by the results of three independent experiments. First, signaling efficiency (agonist-induced *FUS1–lacZ* reporter expression) was attenuated to a similar extent when cells expressed only the mutant receptor at normal levels, and when they coexpressed wild-type receptors at normal levels and mutant receptors at elevated (10-fold) levels (Figure 3a). This result suggested that hetero-oligomers containing mutant and wild-type receptors have reduced activity that is similar to oligomers consisting only of mutant receptors. Second, a G protein sequestration mechanism was unlikely

Figure 3



Attenuation of signaling upon overexpression of a dominant-interfering α-factor receptor mutant. Responses of cells to α-factor (growth arrest and gene expression) were used to quantify the signaling efficiencies. **(a)** For assays of *FUS1–lacZ* expression by β-galactosidase activity, cells expressed normal levels of either wild-type α-factor receptors (WT) or the dominant-interfering mutant receptor (M250I), or they coexpressed wild-type receptors at normal levels and the indicated mutant receptors at elevated levels. **(b–j)** For growth arrest assays, cells expressed normal levels of wild-type α-factor receptors and G protein subunits (WT; b–d), normal levels of wild-type α-factor receptors and overexpressed G protein subunits (WT + $G_{\alpha\beta\gamma}$; e–g), or normal levels of a functional α-factor receptor–G_α subunit fusion (WT–G_α; h–j). Cells also overexpressed wild-type receptors (WT; b,e,h), a dominant-interfering mutant receptor (M250I; c,f,i), or a dominant-interfering mutant receptor with defective G protein coupling activity (M250I/L236R; d,g,j). Defects in cell responsiveness were quantified using various doses of agonist (α-factor); response to a single dose of agonist (1.5 nmol) is shown.

because a dominant-interfering phenotype (attenuation of agonist-induced growth arrest and/or reporter gene expression) was still observed when: the ability of the mutant receptor to interact with G proteins was disrupted (by introducing the L236R substitution in *cis* in its third cytoplasmic loop which we previously showed does not affect agonist binding affinity or receptor expression [13]; Figure 3a,d,g,j); G protein α, β and γ subunits were overexpressed approximately 10–20-fold from a high-copy plasmid carrying the genes encoding each subunit expressed from their normal promoters (Figure 3e–g); and the wild-type receptor was fused to the G_α subunit (Figure 3h–j). Third, overexpression of mutant receptors (10-fold from the *PGK1* promoter) blocked FRET between CFP- and YFP-tagged tailless wild-type receptors (data not shown) without affecting expression of tagged receptors, as indicated by quantitation of CFP and YFP fluorescence and fluorescence microscopy. Therefore, we suggest that oligomerization of α-factor receptors may facilitate signaling.

What might the role of receptor oligomerization in G protein activation be? Oligomerization might stabilize the activated receptor so that each receptor subunit can activate a G protein heterotrimer. An alternative that we currently favor is that receptor subunits in an oligomer contact different subunits of the G protein heterotrimer, possibly providing an efficient means of tilting G_α away from $G_{\beta\gamma}$, leading to GDP release [1,14]. This hypothesis is supported by indications that the cytoplasmic surface of a rhodopsin monomer is only about half the size of the rhodopsin-binding surface of its G protein, transducin [2,15–17]. Furthermore, there is evidence that receptors contact the carboxyl termini of both the G_α and G_γ subunits [18,19]. Therefore, an agonist-activated receptor dimer or oligomer may provide a surface that stabilizes the G protein in a conformation that facilitates GDP release and allows subsequent GTP binding. Because oligomerization may impact several aspects of GPCR function, it would not be surprising if defects in receptor oligomerization are associated with human disease. Pharmacological evidence suggests that schizophrenics may have reduced levels of D2 dopamine receptor dimers [20]. Therefore, studies of diseases that affect various types of G protein signaling pathways may further elucidate the functions of GPCR oligomerization.

Supplementary material

Supplementary material including additional methodological detail and discussion is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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