Oligomerization, Biogenesis, and Signaling Is Promoted by a Glycophorin A-like Dimerization Motif in Transmembrane Domain 1 of a Yeast G Protein-coupled Receptor*

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G protein-coupled receptors (GPCRs) can form dimeric or oligomeric complexes in vivo. However, the functions and mechanisms of oligomerization remain poorly understood for most GPCRs, including the α-factor receptor (STE2 gene product) of the yeast Saccharomyces cerevisiae. Here we provide evidence indicating that α-factor receptor oligomerization involves a GXXXG motif in the first transmembrane domain (TM1), similar to the transmembrane dimerization domain of glycophorin A. Results of fluorescence resonance energy transfer, fluorescence microscopy, endocytosis assays of receptor oligomerization in living cells, and agonist binding assays indicated that amino acid substitutions affecting the glycine residues of the GXXXG motif impaired α-factor receptor oligomerization and biogenesis in vivo but did not significantly impair agonist binding affinity. Mutant receptors exhibited signaling defects that were not due to impaired cell surface expression, indicating that oligomerization promotes α-factor receptor signal transduction. Structure-function studies suggested that the GXXXG motif in TM1 of the α-factor receptor promotes oligomerization by a mechanism similar to that used by the GXXXG dimerization motif of glycophorin A. In many mammalian GPCRs, motifs related to the GXXXG sequence are present in TM1 or other TM domains, suggesting that similar mechanisms are used by many GPCRs to form dimers or oligomeric arrays.

G protein-coupled receptors (GPCR),† which mediate the biological effects of many hormones, neurotransmitters, chemokines, and sensory stimuli, are the largest class of membrane-bound receptors. Although hundreds of GPCRs are encoded by the human genome, their structural and functional diversity is probably greater due to the recently appreciated ability of these receptors to form homo- or heterodimeric/oligomeric complexes in vivo (1–5). However, the mechanisms and functions of GPCR oligomerization remain incompletely understood in part because receptor point mutants defective in oligomerization have not been identified and characterized. Nevertheless, evidence suggests that oligomerization is important for GPCR trafficking to the plasma membrane (6–9), agonist binding activity (10–12), signal transduction (13, 14), and/or receptor down-regulation (15, 16).

Assembly of GPCR dimers/oligomers appears to involve several types of molecular contacts (reviewed in Refs. 3, 4, and 17). Crystallographic studies have revealed that the extracellular N-terminal agonist-binding domain of mGluR1 forms a disulfide-bonded dimer (18), and biochemical evidence indicates that the C-terminal cytoplasmic tails of GABABR1 and GABABR2 interact via non-covalent coiled-coil motifs (19). TM6 may provide a contact site in β-adrenergic receptor dimers because a synthetic peptide corresponding to this region inhibits receptor dimer formation as detected by SDS-PAGE (13, 20, 21). In other receptors, TM1 may provide a dimer or oligomer contact site, as suggested by computational studies of μ-opioid receptors (22) and atomic force microscopy and surface-area modeling of rhodopsin (23). Dimer/oligomer interfaces involving TM4-TM4 and/or TM4/5-TM4/5 contacts have also been suggested for dopamine D2 receptors (24), rhodopsin (22), and γ-opioid receptors (22).

Based on the preceding lines of evidence, various classes of GPCRs may differ in the TM domains they use to form a dimer interface. Alternatively, a given GPCR may have several contact sites involving multiple TM domains, resulting in the formation of oligomeric arrays. Indeed, intersubunit cysteine cross-linking studies of C5a receptors have led to the hypothesis that TM1 provides a dimer contact site, whereas an interface involving TM4/5 is used to assemble oligomeric arrays (25). A variation of this mechanism has been suggested by atomic force microscopy and modeling studies of rhodopsin, which suggest that TM4/5 forms the dimer interface and TM1 is used to assemble dimers into oligomeric arrays (23). Taken together, these findings suggest that GPCRs of different classes may be segregated into separate arrays because different TM domain contacts are used to stabilize dimer and oligomer interfaces.

To define the mechanisms and functions of GPCR oligomerization, we have studied the G protein-coupled α-factor receptor of the budding yeast Saccharomyces cerevisiae (STE2 gene product) as a genetically and biophysically tractable model. We showed initially by using fluorescence resonance energy transfer (FRET) experiments with intact cells or membrane fractions that this GPCR is constitutively dimeric/oligomeric in vivo (1). Subsequent FRET studies of α-factor receptor deletion mutants suggested that TM1 is an important dimerization/
oligomerization interface, which may also involve the extracellular N terminus and TM2 (26).

Here we have explored the functions and mechanisms of GPCR oligomerization in vivo by characterizing oligomerization-defective point mutants of the α-factor receptor. The results suggest that a GXXG motif in TM1 resembling the well-characterized transmembrane dimerization domain of glycophorin-A may be involved in oligomerization and that oligomerization promotes receptor biogenesis and signaling. Because similar sequence motifs are present in TM1 or other TMs of many mammalian GPCRs, the results obtained may be applicable toward understanding the mechanisms of dimer/oligomer formation by a variety of receptors.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—The S. cerevisiae strains used in this study were KBY58 (MATα ura3-52 leu2-3,112 his3-Δ1 trp1 bar1 Δ (27) and NE114-6A (MATα ura3-52 leu2-3,112 his3-Δ1 trp1 sat2 tsc1 ste2A LEU2 (28)), which lacks the RGS protein encoded by the SST2 gene. Deletion of the α-factor receptor structural gene (STE2) in these strains ensured that various receptor mutants expressed from plasmids were the only receptors present in cells. Unless stated otherwise, receptor constructs used in this study lacked sequences encoding the C-terminal cytoplasmic tail, which is dispensable for agonist binding and signaling but is required for receptor internalization and desensitization (28). Use of receptors lacking their C-terminal domains was required to detect FRET between co-expressed CFP- and YFP-tagged α-factor receptors, because the size and/or flexibility of the C-terminal domains of full-length receptors prevents detection of FRET (1, 29). Homophilic interaction between receptor point mutants was studied by co-expression of receptors from their native STE2 promoter on the high copy plasmids pRS423 STE2-tail-YFP and pRS424 STE2-tail-CFP (29). Receptor mutants used for assays of agonist binding and signaling retained the full-length C-terminal tail and were expressed from the native STE2 promoter on a single copy plasmid pRS151 STE2–3myc (30). All mutations were created by use of the Strategene QuickChange™ site-directed mutagenesis kit. The wild type receptor used in endocytosis assays of receptor oligomerization (1) was overexpressed from the constitutive PGK promoter on the high copy plasmid (31).

Fluorescence—Staining fluorescence of intact viable yeast cells co-expressing CFP- and YFP-tagged wild type or mutant α-factor receptors was used to detect FRET between oligomerized receptors in vivo, as described in detail previously (1, 29). Briefly, cells were suspended in 25 mM Tris-Cl, pH 6.8, and irradiated in 3-m glass cuvettes. Cells were irradiated at 425 nm to excite CFP and fluorescence emission was recorded from 450 to 610 nm, or irradiated at 510 nm to excite YFP and fluorescence emission was recorded from 520–610 nm. The FRET acceptor component of the emission spectrum obtained from cells co-expressing CFP- and YFP-tagged receptors was obtained by subtracting components due to CFP emission and YFP emission produced by direct excitation at 425 nm. The apparent efficiency of FRET was calculated by dividing the integrated FRET spectrum by the integrated emission spectrum obtained upon direct excitation of YFP (510 nm; emission recorded from 520 to 610 nm).

Pheromone Response and Agonist Binding Assays—Quantitative assays of agonist-induced growth arrest (halo assays) were used to analyze receptor function (32). The responsiveness of cells to pheromone were KBY58 (α-factor) expressing YFP-tagged wild type or mutant forms of tailless α-factor receptors were grown to mid-log phase, harvested, and lysed on ice by agitation with glass beads in buffer A (10 mM PIPES, pH 6.0, 1 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, and a protein inhibitor mixture). Proteins were resolved by SDS-PAGE (8% acrylamide containing 8% urea), transferred to nitrocellulose, and blocked in 3% non-fat milk plus 3% bovine serum albumin in PBS (phosphate-buffered saline containing 0.1% Tween 20). Blots were incubated with a rabbit anti-GFP polyclonal antibody (gift of P. Silver, Harvard Medical School) diluted 1:2000 in PBS containing 3% skimmed milk albumin. Blots were washed and incubated with a goat anti-rabbit IgG conjugated to horseradish peroxidase (ICN Biomedicals, Inc.) diluted 1:2000 in PBS. After blots were washed, proteins were visualized by enhanced chemiluminescence detection (Amersham Biosciences).

RESULTS

A Glycophorin A-like Dimerization Motif in TM1 of the α-Factor Receptor—Because our previous studies indicated that TM1 is critical for α-factor receptor oligomerization (26), the purpose of the present study was to elucidate the mechanism by which TM1 promotes oligomerization by identifying point mutations that affect oligomerization in vivo. Besides providing insight into the mechanistic principles of α-factor receptor oligomerization, such mutants could also be used to assess effects on receptor biogenesis and function.

Inspection of the TM1 sequence revealed a motif (S2 AIMFGVRCGGAAL26) similar to the GXXG motif within the single transmembrane domain of glycophorin A that mediates dimerization of this membrane protein (35, 36). This sequence motif in the α-factor receptor may be functionally important because saturation mutagenesis of TM1 has revealed that the two glycine residues are intolerant to substitution, in contrast to other regions of TM1 that are tolerant to a variety of amino acid substitutions (37).

By modeling TM1 as an α-helix (37), consistent with structural analyses of TM1 peptides (38, 39), we compared the potential structure of this motif with that of the glycophorin A transmembrane domain (Fig. 1A) determined by NMR (40, 41). In the TM1 model and the glycophorin A transmembrane domain, the two glycine residues of the GXXG motif form a groove on one side of the helix. Immediately adjacent to the groove of glycophorin A is a ridge of four bulky hydrophobic amino acids. Likewise, in TM1 of the α-factor receptor, three of four putative ridge residues have bulky side chains. In the glycophorin A dimer (Fig. 1A), the ridge residues of one subunit pack into the glycine groove of the other subunit, forming an extensive interface stabilized by van der Waals interactions. The GXXG motif in TM1 of the α-factor receptor may be available to form a dimer/oligomer interface because mutagenesis and modeling studies suggest a receptor structure in which these residues are oriented toward the lipid rather than TM domains in the receptor monomer (Fig. 1B) (42).

Mutations Affecting the Glycine Residues in the Glycophorin A-like Motif in TM1 Impair α-Factor Receptor Oligomerization and Biogenesis—We used information from mutagenesis of the glycophorin A dimerization motif to direct our studies aimed at determining whether the GXXG motif in the α-factor receptor is involved in oligomerization. In glycophorin A, the glycine residues forming the groove are critical for dimerization. Substitution of either glycine residue with alanine significantly impairs dimerization, and replacement of a glycine residue with a bulky hydrophobic amino acid such as leucine more strongly disrupts dimerization (43–45). In contrast, single alanine substitutions affecting residues of the ridge have less dramatic effects, probably because the remaining residues of
Accordingly, we targeted the glycine residues (Gly56 and Gly60) predicted to form the groove of the GXGG motif in TM1 of the yeast alpha-factor receptor, and we compared the ability of CFP- and YFP-tagged wild type and mutant receptors to self-associate in FRET experiments employing intact cells. The results indicated that receptors in which Gly56 or Gly60 was replaced by alanine displayed a significantly reduced ability to self-associate in vivo (FRET efficiency 6.4 ± 2.0% and 7.5 ± 1.6% for G56A and G60A mutants, respectively; Fig. 2A and Table I), as compared with the ability of wild type receptors to self-associate (FRET efficiency 12.4 ± 1.8%; Fig. 2A and Table I). The effects of these mutations on FRET efficiency were not due to significant differences in protein expression as indicated by immunoblotting of whole cell extracts (Fig. 2B). However, plasma membrane targeting of the glycine → alanine single mutant receptors was reduced 3–6-fold as indicated by the results of agonist binding assays using intact, inviable cells (Table I and data not shown). The results of these experiments also indicated that mutant receptors were not severely misfolded because their agonist binding affinities were similar to that of wild type receptors (Table I). Fluorescence microscopy revealed that GFP-tagged tailless mutant receptors were retained in the ER to a greater extent than wild type tailless receptors (Fig. 3), whereas full-length GFP-tagged mutant receptors displayed reduced cell surface expression and increased accumulation in endosomes and the lysosome-like vacuole as shown by co-staining with FM 4-64 (Fig. 3 and data not shown). However, ER targeting defects were unlikely to explain the reduced efficiency of FRET observed with tailless mutant receptors, because we have shown previously (26) that wild type receptors self-associate with similar efficiency (FRET efficiency) in ER and plasma membrane fractions. Therefore, the present results suggested that oligomerization is required for efficient targeting of the alpha-factor receptor to the plasma membrane.

Consistent with this hypothesis, we observed more dramatic
cell surface expression defects with mutant receptors in which both glycine residues were replaced by alanine (G56A,G60A). This double mutant receptor was severely impaired in the ability to self-associate as indicated by a FRET efficiency (4.9/1000 1.0%; Fig. 2 and Table I) near the nonspecific background established in our previous studies (2–3% (26)). Furthermore, whereas this double mutant receptor was expressed normally at the protein level (Fig. 2B), it strongly mislocalized to the ER (tailless mutant) and the vacuole (full-length mutant) (Fig. 3). Consistent with these results, agonist binding assays revealed that cell surface expression of this mutant receptor was reduced 20-fold (Table I). However, the double mutant receptor had relatively normal agonist binding affinity, again indicating that mutations affecting the glycine residues of the GXXXG motif did not result in receptors that were grossly misfolded. Therefore, these results reinforced the conclusion that oligomerization is required for efficient biogenesis of the α-factor receptor.

To provide an independent means of determining whether mutations affecting the glycine residues of the GXXXG motif in TM1 impair α-factor receptor oligomerization, we determined whether mutant receptors could interact with wild type receptors during endocytosis by using an assay we developed previously (1, 26). This assay relies on the observation that overexpressed untagged wild type receptors can associate with endocytosis-defective tailless receptors tagged with GFP, thereby recruiting GFP-tagged tailless receptors into endosomes. This assay is specific because other GFP-tagged membrane proteins that normally fail to undergo endocytosis are not recruited into the endocytic pathway upon overexpression of wild type α-factor receptors (1).

Endocytosis assays of receptor oligomerization were performed using cells that co-expressed untagged full-length wild type receptors with YFP-tagged tailless receptors in which either one or both of the glycine residues in TM1 were changed to alanine. As a control, we demonstrated the ability of YFP-tagged tailless wild type receptors to interact with untagged full-length wild type receptors, as indicated by the appearance of YFP-labeled endosomes, agonist-stimulated clearance of YFP-tagged tailless receptors from the plasma.

Fig. 2. Use of FRET to determine the efficiency of homo-oligomerization of receptors bearing mutations in the GXXXG motif of TM1 in vivo. A, FRET experiments to detect the ability of CFP- and YFP-tagged forms of the indicated tailless receptors to self-associate in living yeast cells. FRET data were collected and analyzed as described under “Experimental Procedures.” Each panel shows four emission spectra obtained upon excitation of cells at the λmax for CFP as follows: one spectrum from cells co-expressing CFP- and YFP-tagged forms of the indicated receptor (dotted plus dashed line); a second spectrum from cells expressing only the indicated CFP-tagged receptor (dotted line); a third spectrum from cells expressing only the indicated YFP-tagged receptor (dashed line); and a fourth spectrum that shows the resultant fluorescence emission due specifically to FRET (solid line) in cells co-expressing CFP- and YFP-tagged receptors. Emission due to FRET was determined by subtracting the second and third emission spectra from the first spectrum, as described under “Experimental Procedures.” The efficiency of FRET was determined by dividing the integrated FRET spectrum by the integrated YFP emission spectrum obtained by exciting cells co-expressing CFP- and YFP-tagged receptors at the λmax of YFP. All tagged receptors were expressed from plasmids in cells carrying a deletion of the chromosomal gene encoding the α-factor receptor. Each experiment was performed at least three times in duplicate; results of a single representative experiment are shown. WT, wild type. B, expression of the indicated YFP-tagged receptors used for FRET experiments as detected by probing immunoblots of whole cell extracts with GFP antibodies. Cells that did not express a YFP-tagged receptor were used as a control (—).
Oligomerization efficiency was determined by FRET and endocytosis assays as described under “Experimental Procedures.” FRET efficiencies were calculated as the average of results from at least three independent transformants assayed in duplicate. Endocytosis efficiencies were calculated as the percentage of cells containing endosomes labeled with tail-less GFP-tagged forms of the indicated receptors under conditions where wild type untagged receptors were co-expressed, and cells were treated 1 h with agonist (5 μM). Agonist binding data are the average of results obtained from Scatchard analyses of at least three independent transformants of each mutant assayed in triplicate. ND, not detected; –, not assayed.

| Receptor | Apparent FRET efficiency | Endocytosis efficiency | $K_d$ | $B_{max}$/sites/cell |
|----------|--------------------------|------------------------|-------|---------------------|
| WT       | 12.4 ± 1.8               | 93.6                   | 2.1 ± 1.4 | 7930 ± 203 |
| G56A     | 6.4 ± 2.0                | 24.4                   | 0.5 ± 0.1 | 1200 ± 266 |
| G60A     | 7.5 ± 1.6                | 43.7                   | 4.7 ± 1.2 | 2410 ± 656 |
| G56A,G60A| 4.9 ± 1.0                | 6.9                    | 3.2 ± 1.7 | 404 ± 97 |
| G56L     | 3.5 ± 1.0                | ND                     | ND     | ND |
| G60L     | 3.3 ± 1.9                | ND                     | ND     | ND |
| G56L,G60L| 2.5 ± 1.5                | ND                     | ND     | ND |
| I53A     | 10.0 ± 0.6               | –                      | –      | – |
| V57A     | 11.3 ± 0.5               | –                      | –      | – |
| L64A     | 11.8 ± 0.7               | –                      | –      | – |

Fig. 3. Subcellular localization of α-factor receptor mutants bearing substitutions affecting the GXXXG motif in TM1. A. Fluorescence microscopy of cells expressing GFP-tagged tailless wild type (WT) and mutant receptors bearing the indicated mutations affecting the GXXXG motif in TM1. B. Fluorescence microscopy of cells expressing GFP-tagged full-length wild type and mutant receptors bearing the indicated mutations affecting the GXXXG motif in TM1. All tagged receptors were expressed from plasmids in cells carrying a deletion of the chromosomal gene encoding the α-factor receptor.

In contrast, cells co-expressing untagged wild type receptors and tailless YFP-tagged mutant receptors bearing glycine to alanine substitutions at either or both positions in TM1 exhibited significantly reduced labeling of endosomes (Fig. 4 and Table I). Furthermore, the double mutant tailless receptor (G56A,G60A) exhibited a more severe defect (7% cells contained labeled endosomes; Table I) than either single mutant (24 and 44% of G56A and G60A cells, respectively, contained endosomes; Table I), in accord with their relative oligomerization defects as indicated by FRET experiments. The results of these experiments also indicated that the single and double mutant receptors failed to undergo efficient agonist-induced endocytosis as indicated by the relatively sustained appearance of tagged receptors on the cell surface and failure to accumulate significantly in the vacuole following treatment with α-factor (Fig. 4). Therefore, we conclude that glycine to alanine substitutions in the GXXXG motif of TM1 greatly impair oligomerization of the α-factor receptor.

Oligomerization Promotes α-Factor Receptor Signaling—To address whether oligomerization promotes receptor signaling, we analyzed the ability of oligomerization-defective mutants described above to respond to agonist. Mutations affecting the GXXXG motif were likely to be useful because they preserve the agonist binding affinity of the receptor, and their modest effects on receptor cell surface expression were unlikely to confound the analysis of signaling assays because previous studies have demonstrated that α-factor receptor levels can be reduced 20-fold without affecting the efficiency of signaling (46).

Accordingly, we performed assays of agonist-induced cell cycle arrest using cells expressing wild type or mutant receptors in which either or both glycine residues in TM1 were replaced by alanine. Cells expressing the G60A mutant from its normal promoter on a single copy plasmid exhibited a nearly normal signaling phenotype, as indicated by the formation of a zone of growth inhibition that was only slightly smaller than that obtained with wild type controls (Fig. 5A). In contrast, cells expressing the G56A single mutant or the G56A,G60A double mutant from their normal promoter on single copy plasmids exhibited greatly impaired responses to agonist relative to wild type receptor controls, as indicated by the formation of smaller/turbid zones of growth inhibition (Fig. 5A). These signaling defects were not corrected significantly by overexpression of the G56A or G56A,G60A mutant receptors from high copy plasmids (Fig. 5A), which increased receptor cell surface...
expression 3–5-fold as indicated by the results of agonist binding assays (data not shown). Therefore, the signaling phenotypes of these receptor mutants were unlikely to be caused by impaired cell surface expression. These results provided an initial indication that oligomerization facilitates α-factor receptor signaling.

As a further means of addressing whether oligomerization promotes α-factor receptor signaling, we determined whether the magnitudes of these signaling defects of these mutants correlated with the oligomerization defects revealed by FRET experiments (Table I). Taken together, these results reinforce the hypothesis that oligomerization is important for α-factor receptor signaling.

**Mechanism of Oligomerization Mediated by the GXXXG Motif in TM1 of the α-Factor Receptor**—Our final objective was to determine whether the glycophorin A-like motif in TM1 of the α-factor receptor promotes oligomerization by a mechanism similar to that used by the well characterized dimerization motif in glycophorin A. We addressed this objective by performing structure-function experiments that took advantage of previous studies of glycophorin A. These studies of glycophorin A showed that substitution of either glycine residue in the GXXXG motif with a bulky hydrophobic residue such as leucine causes a stronger dimerization defect than a glycine to alanine substitution (48), apparently because of steric clash.

Consistent with these principles, we found that substitution of either one or both glycine residues in the GXXXG motif in TM1 of the α-factor receptor with leucine resulted in a FRET signal near the level of nonspecific background (Fig. 2A and Table I) and caused a strong signaling defect (failure to form detectable agonist-binding sites on the cell surface (Fig. 3) and by the lack of detectable agonist-binding sites on the cell surface (Table I). These results therefore were consistent with the hypothesis that TM1 promotes oligomerization by a mechanism similar to that used by the dimerization domain of glycophorin A.

In contrast to the critical roles of the glycine residues that form the groove of the transmembrane dimerization domain of glycophorin A, individual residues forming the ridge that inserts into the groove have less important roles because alanine substitutions affecting the ridge have little effect on dimerization (43–45). Apparently, a given residue in the ridge is not essential for dimerization of glycophorin A because van der
Waals contacts made by the remaining residues of the ridge are sufficient to stabilize the dimer.

To determine whether the same principles govern oligomerization of the α-factor receptor, we constructed and analyzed a collection of single mutants in which each residue predicted to form the ridge (Fig. 1A) was replaced with an alanine (I53A; V57A; L64A). Compared with wild type receptors, each mutant receptor self-associated, localized, and signaled normally, as indicated by FRET (Fig. 6 and Table I), fluorescence microscopy of GFP-tagged receptors, and agonist-induced growth arrest assays (data not shown). Therefore, the GXXG motif in TM1 of the α-factor receptor appears to mediate oligomerization by a mechanism similar to that used by the transmembrane dimerization motif of glycophorin A.

**DISCUSSION**

By analyzing mutations affecting a GXXG motif in TM1 similar to the transmembrane dimerization domain of glycophorin A, we have provided evidence indicating that oligomerization is required for biogenesis of and signaling by a G protein-coupled receptor (α-factor receptor; STE2 gene product) of the yeast *S. cerevisiae*, and we have provided insight into the oligomerization mechanisms of this receptor. Amino acid substitutions affecting the GXXG motif impaired oligomerization and trafficking of α-factor receptors to the cell surface but did not significantly affect agonist-binding affinity. Analysis of the signaling activities of these mutant receptors indicated that the magnitudes of the oligomerization and signaling defects were correlated. Although mutant receptors displayed partial defects in cell surface targeting, these trafficking defects were unlikely to account for the signaling defects observed. We suggest this because decreasing expression of wild type receptors to a similar degree (up to 20-fold) does not impair signaling (46), and because overexpression of oligomerization-defective receptors from high copy plasmids increases cell surface expression but does not correct the signaling defects. Therefore, oligomerization promotes efficient biogenesis of and signaling by α-factor receptors.

Does the GXXG motif in TM1 provide the major sequence feature that promotes α-factor receptor oligomerization? Although further investigations are necessary to answer this question, the studies of Dumont and colleagues (37) suggest that this may be the case. These investigators used saturation mutagenesis of all seven TM domains to identify amino acid residues required for α-factor receptor function (37). This approach revealed that the glycine residues in the GXXG motif of TM1 are especially important for receptor function, because the only substitutions of these residues that preserve detectable levels of receptor function are alanine or serine. This is in striking contrast to other sequences in TM1 or other TM domains, which permit a much wider range of allowable amino acid substitutions (37). Therefore, these findings in conjunction with our demonstration that oligomerization promotes α-factor receptor signaling suggest the hypothesis that the GXXG motif in TM1 is a primary determinant of α-factor receptor oligomerization.

**Functions of Oligomerization in α-Factor Receptor Signaling**—Why does an oligomerization defect impair signaling by α-factor receptors? On the one hand, oligomerization may promote agonist-induced conformational changes in the receptor. Indeed, recent studies (49) of chimeric GABAB receptors suggest that heterodimerization is required for efficient allosteric activation of this receptor. Alternatively, oligomerization of the α-factor receptor may be required to generate a cytoplasmic surface large enough to interact with and activate G protein heterotrimers. However, this hypothesis seems unlikely because structural studies of rhodopsin and transducin indicate that the cytoplasmic surface of a GPCR is sufficient to occupy the receptor interaction surface of a G protein heterotrimer (50, 51). Furthermore, recent studies indicate that only one subunit of the GABA<sub>B</sub>R1-R2 heterodimeric complex activates the G protein (52). A third possibility is that oligomerization is not required for G protein activation per se but instead is important for signal amplification. Amplification may occur if receptors associate as large arrays such that G protein heterotrimers are activated efficiently in a spatially restricted manner, leading to efficient, localized downstream signaling. Further studies of α-factor receptors are underway to address these hypotheses.

**Oligomerization and GPCR Trafficking**—The retention of oligomerization-defective tailless α-factor receptors in the endoplasmic reticulum and the accumulation of oligomerization-defective full-length α-factor receptors in the vacuole are probably due to quality control processes analogous to those used to regulate expression of other oligomeric membrane proteins (53, 54). The mechanism by which mutant α-factor receptors are retained in the ER remains to be established because the signal(s) in the receptor that mediates this process are unknown. Nevertheless, retention signals are relatively degenerate in sequence and are present within several transmembrane domains of the receptor because truncation mutant receptors lacking any of several transmembrane domains are retained in the ER (26). This is in contrast to GABA<sub>B</sub>R2 which contains a distinct ER retention motif in its C-terminal cytoplasmic tail, which upon heterodimerization with GABA<sub>B</sub>R1 is masked such that the heterodimeric complex traffic to the plasma membrane (8).

**α-Factor Receptor Oligomerization Mechanisms**—Although the GXXG motif in TM1 appears to mediate oligomerization of the α-factor receptor, does this motif directly form the contact site or does it indirectly mediate oligomerization by affecting the structure of TM1? The direct contact model is supported by several observations. First, our previous studies of α-factor receptor deletion mutants have shown that a fragment contain-
Fig. 7. Presence of a GXXGX motif or its variants in TM1 in subfamilies of GPCRs. Helical bundles were created with the consensus sequence of each class of GPCR using data obtained from the GPCR data base (www.gpcr.org). The N terminus of each TM helix is at the top and C terminus at the bottom. The upper row contains GPCR subfamilies in which the TM1 consensus sequence contains a sequence that matches a canonical GXXGX motif or variants defined by the consensus (AGSTP)(XXX)(GAS) (indicated in black), as discussed in the text. The lower row contains GPCR subfamilies that lack a GXXGX variant motif but that contain at least one glycine residue (indicated in black). The single letter amino acid code is used.

Table II

GXXGX motifs in various TM1 of representative GPCRs

| Receptor     | TM   | TM sequence                          |
|--------------|------|--------------------------------------|
| Gpa dimerization motif | I    | ...LIXXXGXXXGXXXT...                |
| Ste2 S. cerevisiae (48–72) | I    | TVQAAGVRGAGCaALTLIVGMHI            |
| Ste2 S. bayanus (48–72) | I    | TVQAAGVRGAGCaALTLIVGMHI            |
| Ste2 S. mikatae (48–72) | I    | TVQAAGVRGAGCaALTLIVGMHI            |
| Ste2 S. kudriavzevii (48–72) | I    | KITAAGVRGAGCaALTLIVGMHI            |
| Ste2 S. castellii (48–72) | I    | KITAAGVRGAGCaALTLIVGMHI            |
| ml-ACh human (24–48) | I    | QVAFIGTGTLSLAVTIVGNI                |
| Ste3 S. cerevisiae (71–94) | III   | IIVKLQGANIGICAVTN1IYNL             |
| OP57 human (110–131) | III   | EGFLQTVNIAGVNLALAFE                |
| CCR2 human (107–114) | III   | LFTGLHITYGGGQFGFPLTTLIDF           |
| GLR human (72–293) | IV    | FFSFLQGKGAMPHFLEVPVNAQV            |
| NY2R human (164–187) | IV    | ISPLIGTIGALIGSALLASPAIIRF          |
| PE21 human (308–333) | IV    | VEMQVQAVGVMVSCICSMMFVLLVA          |
| OLF2 human (200–221) | V     | LVIFAFAGGVQSTSPF3CILSLYSR          |
| β2-AR human (272–296) | VI    | LKTGILMSTFILCLWLPFFIVNIVW          |
| β4-AR human (291–303) | VI    | RAIVGTINGFILCWLPFFILANLV           |
| α1-A-AR human (340–363) | VI    | KAAKTVGLVIGCIFLWLPFFIALPL          |
| 5H1B human (343–368) | VI    | KATIKQLIGAFFIVWLPFFISL             |
| SCRC rat (374–396) | VII   | FEALIGFQGLGQALVYFCFLNSEQV          |
tors when co-expressed in yeast (60). Accordingly, we suggest that GXXG-like motifs may be able to direct the formation of specific GPCR complexes as follows. 1) GXXG motifs in various GPCRs are located in different TM domains. 2) Residues flanking the GXXG motif are known to affect binding affinity (61). 3) Residues forming the ridge that inserts into the glycosine groove of the GXXG motif are known to affect additional binding energy (62). 4) GXXG motifs interact efficiently when present at a similar depth in the membrane as determined by their position within the linear sequence of the TM domain.

However, other motifs in TM domains may also be crucial for GPCR oligomerization. Indeed, leucine-rich or serine/threonine-rich motifs are known to drive assembly of dimers/oligomers in other transmembrane proteins (62–67). Furthermore, the formation of specific dimers/oligomers is likely in several GPCRs to involve non-TM domain contacts (reviewed in Refs. 3, 4, and 17), such as the extracellular N-terminal domain (8). Thus, in conclusion, further studies of the α-factor receptor and other receptors will reveal whether GXXG motifs, or variants, or other motifs provide the major mechanisms by which GPCRs form specific dimers and oligomers. This information will also reveal the functions of oligomerization in the processes of GPCR biogenesis, localization, turnover, and signaling.

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