Assembly of G Protein-Coupled Receptors from Fragments: Identification of Functional Receptors with Discontinuities in Each of the Loops Connecting Transmembrane Segments†

Negin P. Martin, LuAnn M. Leavitt, Christine M. Sommers, and Mark E. Dumont*

Department of Biochemistry and Biophysics, P.O. Box 712, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received August 25, 1998; Revised Manuscript Received November 2, 1998

ABSTRACT: The α-factor receptor of the yeast *Saccharomyces cerevisiae* is a member of the superfamily of G protein-coupled receptors that mediate signal transduction in response to sensory and chemical stimuli. All members of this superfamily contain seven predicted transmembrane segments. We have created a series of genes encoding α-factor receptors with amino- or carboxyl-terminal truncations at each of the loop regions connecting transmembrane segments. Split receptors containing a discontinuity in the peptide backbone were synthesized by coexpressing pairs of truncated receptor fragments in yeast. Complementary pairs of fragments split at sites within each of the cytoplasmic and extracellular loops were capable of assembling and transducing a signal in response to α-factor binding. One pair of noncomplementary fragments containing a deletion in the second intracellular loop of the receptor also yielded a functional receptor. Coexpression of certain combinations of overlapping fragments containing supernumerary transmembrane segments also led to formation of functional receptors, apparently because of proteolytic trimming of overlapping regions. Coexpression of truncated receptor fragments with full-length receptors had no effect on signaling by the full-length receptors. These results demonstrate the following: (1) Correct folding of the α-factor receptor does not require a covalent connection between any pair of transmembrane segments that are adjacent in the sequence. (2) Most of the second intracellular loop of the receptor is not required for function. (3) The structure of the receptor cannot, in most cases, tolerate the presence of extra transmembrane segments. (4) None of the truncated fragments of the α-factor receptor can efficiently oligomerize with normal receptors in such a way as to inhibit receptor function.

Spontaneous folding of proteins into defined three-dimensional structures requires specific intramolecular interactions between different parts of a polypeptide chain. In some cases, these interactions are capable of preserving the structure of the protein in the presence of discontinuities in the polypeptide chain. For example, several structurally well-characterized soluble proteins, such as ribonuclease A (*1*), staphyloccocal nuclease (*2*), and cytochrome *c* (*3*), are capable of assembling from separate pieces of polypeptide chain that are not covalently linked. However, the success of such intermolecular assembly appears to be dependent on choosing particular sites for the polypeptide chain discontinuities that are located between defined structural elements.

The folding of α-helix-containing transmembrane proteins has been proposed to occur via a two-step pathway (*4*). In the first step, transmembrane helical segments form and are translocated across the membrane. In the second step, the transmembrane segments spontaneously associate with each other to form the overall tertiary structure. Consistent with this proposed pathway, a number of membrane proteins including bacteriorhodopsin (*5*, *6*), the *lac* permease of *Escherichia coli* (*7*), several G protein-coupled receptors (*8*−*11*), P-glycoprotein (*12*), the human red cell anion exchanger (*13*), and the *SEC61* protein translocator (*14*) are capable of assembling from noncovalently linked polypeptide chains comprising separate groups of transmembrane segments. In each of these cases, assembly from fragments has previously been reported to occur only when discontinuities in the polypeptide chain reside between particular transmembrane segments. We report here that the α-mating pheromone receptor of the yeast *Saccharomyces cerevisiae*, a G-protein coupled receptor with the seven predicted transmembrane segments typical of proteins of this class (*15*, *16*), can be assembled from complementary pairs of fragments with discontinuities in each of the six loops connecting the transmembrane segments and from a noncomplementary pair of fragments containing a deletion in IC2,[1] the second intracellular loop. We also report that expression of truncated fragments of the α-factor receptor does not interfere with

† This work was supported by Grant VM-169 from the American Cancer Society and a Biomedical Pilot Initiative Grant from the Charles E. Culpeper Foundation.

* Corresponding author: phone 716-275-2466; Fax 716-271-2683; E-mail mdmt@db6.cc.rochester.edu.

[1] Abbreviations: IC1, first intracellular loop; EC1, first extracellular loop; IC2, second intracellular loop, etc.; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; ONPG, *o*-nitrophenyl β-D-galactoside; TM1, first transmembrane segment; TM2, second transmembrane segment, etc.; GPCR, G protein-coupled receptor; HA, hemagglutinin.

10.1021/bi982062w CCC: $18.00
Published on Web 12/22/1998

Table 1: Plasmids Expressing Amino- and Carboxyl-Terminal Fragments of the α-Factor Receptor

amino acid sequence[a]	plasmid	oligonucleotide	Δ(C−N)[b]	predicted NH_2-terminal orientation[c]	yeast strain coexpressing fragment and *STE2*$^{+}$ [d]
M1−T78	pMD429	ON214	+1[c]	exo	A763
M1−T114	pMD430	ON215	+1	exo	A764
M1−V152	pMD428	ON206	+1	exo	A765
M1−T155	pMD456	ON193	+1	exo	
M1−I162	pMD458	ON195	+1	exo	
M1−A198	pMD431	ON216	+1	exo	A766
M1−I230	PMD418	ON164	+1	exo	A768
M1−L236	pMD426	ON199	+1	exo	A767
M1−G273	pMD432	ON217	+1	exo	A769
M1−end	pMD410		+1	exo	A829[e]
P79−end	pMD421	ON188	0	cyto	A771
G115−end	pMD422	ON190	+0.5	exo	A772
I153−end	pMD433	ON165	−3	cyto	A773
G156−end	pMD457	ON194	−3	cyto	
G163−end	pMD459	ON196	−2	cyto	
T199−end	pMD423	ON198	+4	exo	A774
R231−end	pMD434	ON166	−4.5	cyto	A776
G237−end	pMD427	ON200	−1.5	cyto	A775
T274−end	pMD424	ON202	0	exo	A777

[a] Methionine is the amino-terminal residue encoded by all carboxyl-terminal fragments. This residue is not indicated in the nomenclature used in this table. [b] Δ(C−N) is the charge difference between the carboxyl and amino termini of the first predicted transmembrane segment in the receptor fragment. The charge is summed over 15-amino acid windows as described by Hartmann et al. (*52*), including the effect of amino termini introduced by truncation. Three potentially charged residues that reside in the middle of predicted transmembrane regions, Arg58, His94, and Glu143, were not included in these calculations, since the existence of charged groups in the middle of transmembrane segments is not explicitly considered in the algorithm of Hartmann et al., (*52*). Two of these charged residues, Arg58 and Glu143, can be changed to residues with uncharged side chains with minimal effects on receptor function (*24*), and substitution of an uncharged residue for Arg58 had no effect on the topological orientation of the first transmembrane segment expressed as a fusion protein (*53*). [c] This column indicates the predicted location of the amino terminus of the fragment assuming that this location is the same as in the intact α-factor receptor. Exo = extracellular; cyto = intracellular. [d] This column lists strains used for testing the effects of coexpression of fragments with intact receptor (Figure 7). The indicated multicopy plasmid was expressed in strain A230 containing a chromosomal copy of *STE2*$^{+}$. [e] In assays of *FUS1*−*lacZ* induction by strains coexpressing amino-terminal fragments and intact receptors, the yeast strain A829 containing the multicopy *URA3*$^{+}$ plasmid pMD345 encoding c-*myc*-tagged *STE2* was used instead of PMD410 as a *STE2*$^{+}$ control.

signaling by normal full-length receptors expressed in the same cells.

The α-mating pheromone receptor, encoded by the *STE2* gene, is found on the surface of haploid yeast cells of the **a**-mating type. Activation of the receptor by binding of α-factor, a 13-residue peptide secreted by cells of the α-mating type, leads to dissociation of a cytoplasmic heterotrimeric G protein. The liberated β and γ subunits of this G protein activate a MAP kinase phosphorylation cascade that ultimately results in cell cycle arrest, altered transcription of mating-related genes, and changes in cell morphology in preparation for mating of the two haploid cells to form a diploid zygote (*17*−*19*). The pheromone response pathway in yeast resembles mammalian G protein-coupled receptor-mediated pathways responsible for the responses to a wide variety of sensory stimuli, hormones, and neurotransmitters. Mammalian G protein-coupled receptors expressed in yeast are capable of ligand-dependent activation of the yeast pheromone response pathway (*20*, *21*).

The ability to assemble membrane proteins from fragments has provided a useful way of analyzing membrane topology (*7*), identifying helix−helix interactions in transmembrane proteins (*22*, *23*), and localizing sites of intermolecular interactions involved in protein function (*14*). Expression of the yeast α-factor receptor from fragments will allow application of these approaches to understanding the structure and function of this G protein-coupled receptor. In addition, the ease of genetic manipulation of yeast has allowed us to assay for coassembly of a variety of combinations of fragments as a test of the generality with which noncovalent interactions among transmembrane segments can drive folding of a multispan membrane protein.

EXPERIMENTAL PROCEDURES

Plasmids. The multicopy plasmids expressing receptor fragments and the oligonucleotides used to create them are listed in Table 1. Sequences of the oligonucleotides are available on request.

A scaffold plasmid, pMD239, used for the creation of amino-terminal fragments of *STE2* was created from the *STE2* gene derived from a CEN plasmid, pMD149 (*72*). The original influenza hemagglutinin (HA) epitope-tagged *STE2* gene in pMD149 contained four cysteine residues. Two of these were present in the normal *STE2* gene. Two additional cysteine residues had been introduced at the extreme carboxyl terminus in the process of introducing the epitope (*24*). For the eventual use of the receptor fragments for cysteine cross-linking, we wished to remove all cysteine residues from *STE2*. Plasmid pMD194, containing an HA epitope-tagged *STE2* gene with all cysteine residues removed, was derived from plasmid pMD149 by site-directed mutagenesis with oligonucleotides ON54, ON55, ON69, and ON70, converting Cys59 and Cys252 to serine residues and the two additional cysteine residues at the ends of the HA epitope to glycine residues. To allow expression of the amino-terminal fragments on multicopy plasmids, the plasmid pMD239 was created by removing a *Sac*I−*Sph*I fragment containing the cysteine-less *STE2* coding region from plasmid pMD194 and inserting it into *Sac*I−*Sph*I-cut pMD228 (*72*), a vector containing 2μ replication sequences and a *URA3* marker.

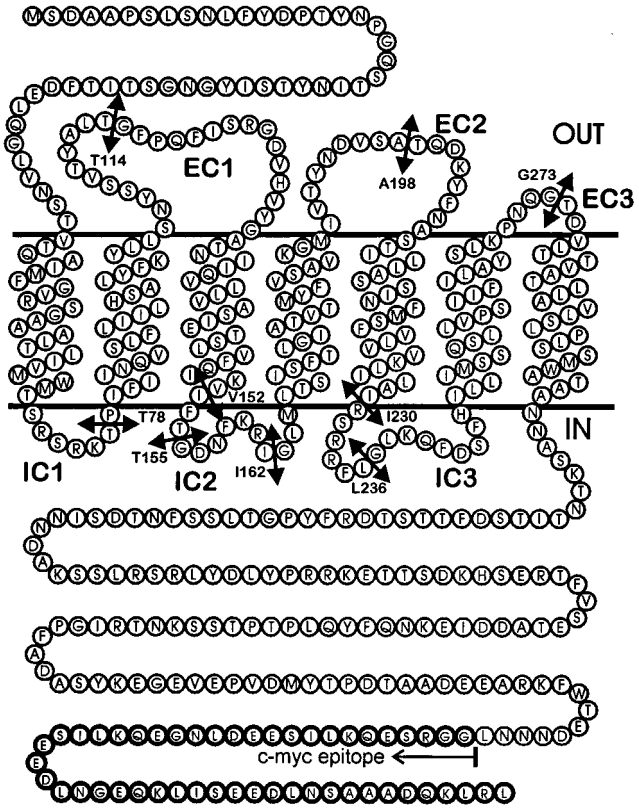

FIGURE 1: End points of truncated receptor fragments. Arrows indicate the locations of end points of receptor fragments, displayed on a diagram of the predicted topological orientation of the seven transmembrane segments of the α-factor receptor. IC1, IC2, and IC3 indicate the locations of the three intracellular loops. EC1, EC2, and EC3 indicate the locations of the three extracellular loops. Amino acid residues outlined in bold circles were added as part of the epitope tag.

Removal of cysteine residues from the *STE2* gene had no effect on receptor function (results not shown). Note that the *STE2*$^+$ allele used encodes lysine at codon 269, as reported in initial sequencing of the gene (*25*) but in contrast to the glutamic acid shown at this position in the *S. cerevisiae* genome database.

The plasmid pMD410 served as a scaffold for creation of carboxyl-terminal fragments of *STE2*. This plasmid contains a *LEU2* marker and a *STE2* gene tagged with a triple c-*myc* epitope (*26*). The c-*myc* epitope was introduced by removing a *Not*I fragment containing the HA epitope from the 3′ end of the cysteine-less *STE2* gene and ligating in a *Not*I fragment containing three tandemly repeated copies of the epitope to obtain plasmid pMD354. The resulting amino acid sequence of the carboxyl terminus of *STE2* is shown in Figure 1. The *Sph*I−*Sac*I fragment containing this cysteine-less c-*myc*-tagged version of *STE2* was then excised from pMD354 and ligated as a replacement for the corresponding *Sph*I−*Sac*I fragment of the multicopy *LEU2*-containing plasmid, pMD267 (*72*) to create pMD410.

yEPlac181, a multicopy *LEU2* vector (*27*), was used as a control plasmid to convert some strains to *LEU2*$^+$.

With one exception (see below), the truncated receptors were created by using oligonucleotide-directed mutagenesis to introduce deletions in plasmids pMD239 or pMD410 by use of single-stranded DNA as described (*19*, *28*). Carboxyl-terminal deletions removed sequences between the codon for the desired carboxyl-terminal amino acid and the termination codon of the HA epitope-tagged *STE2*$^+$ allele (*24*). Amino-terminal deletions removed the sequences between the normal *STE2* initiation codon and the desired amino-terminal residue, thereby introducing a methionine residue as the initial residue in the translation product. In some cases this methionine may subsequently be cleaved (*29*). Compared with truncation by modification of initiation or termination codons, the creation of deletions removes sequence overlap that could lead to recombinational repair of complementary fragments. To enrich the mutagenesis reactions for the desired products, samples were digested with a restriction enzyme that cuts uniquely in the region to be deleted before the reactions were transformed into *E. coli*. Following the mutagenesis reactions, plasmids were isolated from *E. coli*, screened by restriction digests to check for the presence of the deletion, and then subjected to DNA sequencing of the region targeted for mutagenesis. Sequencing of double-stranded plasmids was performed with Sequenase (Amersham Life Sciences) according to the manufacturer's directions.

One receptor fragment truncated at its carboxyl terminus (plasmid pMD418 expressing *STE2* residues 1−230) contained a point mutation (AGA → TGA) introducing a stop codon rather than a true deletion of the carboxyl terminal. However, following prolonged growth on plates, we found no evidence for regain of function indicative of recombination by any of the complementary pairs of truncated receptors, and all carboxyl-terminal fragments exhibited the expected apparent molecular weight when assayed by immunoblotting. Plasmid pMD418 was created by oligonucleotide-directed mutagenesis of the *STE2* gene derived from pMD104 (*24*) with oligonucleotide ON164. The *Sph*I−*Sac*I fragment containing this *ste2* allele was ligated as a replacement for the corresponding fragment in the multiple cloning site of pMD228.

Independent isolates of one strain, A689, expressing overlapping fragments composed of five amino-terminal transmembrane segments and five carboxyl-terminal transmembrane segments exhibited a bimodal distribution of levels of *FUS1*−*lacZ* induction. The better-responding isolates of this strain displayed pheromone responses that were 45−100% of those seen with normal receptors. The more weakly responding strains responded 4−7% as well as normal receptors. High levels of pheromone response correlated with the detection of a major band corresponding to full-length receptor on immunoblots, indicating that the multicopy plasmids encoding the two fragments may have recombined to create a full-length *STE2*$^+$ gene. Thus, for this particular strain, only the pheromone responsiveness of transformants with the lower activity is shown in Figure 6.

To allow expression of the receptor in three complementary fragments, a truncated gene encoding the central five transmembrane segments of the receptor was cloned into a multicopy plasmid containing the gene for G418 (geneticin) resistance. Oligonucleotides ON188 and ON217 were used to introduce the amino- and carboxyl-terminal truncations by site-directed mutagenesis of plasmid pMD239 to create plasmid pMD460. A *Bgl*II fragment containing the *URA3* gene was then excised from pMD460 and replaced by the G418 resistance gene from pUG6 (*30*) to produce plasmid pMD468.

Table 2: Yeast Strains Containing Single Receptor Fragments and Complementary Pairs of Fragments

strain[a]	plasmids	plasmid-encoded regions of *STE2*[b]
A587N	pMD228 + pMD429	M1−T78
A588NC	pMD421 + pMD429	M1−T78 + P79−end
A589C	yEPlac181 + pMD421	P79−end
A590N	pMD228 + pMD430	M1−T114
A591NC	pMD422 + pMD430	M1−T114 + G115−end
A592C	yEPlac181 + pMD422	G115−end
A593N	pMD228 + pMD428	M1−V152
A594NC	pMD428 + pMD433	M1−V152 + I153−end
A595C	yEPlac181 + pMD433	I153−end
A596N	pMD228 + pMD431	M1−A198
A597NC	pMD423 + pMD431	M1−A198 + T199−end
A598C	yEPlac181 + pMD423	T199−end
A599N	pMD228 + pMD426	M1−L236
A600NC	pMD426 + pMD427	M1−L236 + G237−end
A601C	yEPlac181 + pMD427	G237−end
A602N	pMD228 + pMD432	M1−G273
A603NC	pMD424 + pMD432	M1−G273 + T274−end
A604C	yEPlac181 + pMD424	T274−end
A607WT	pMD228 + pMD410	M1−end
A608NR	pMD228 + yEPlac181	*ste2*−Δ
A638N	yEPlac181 + pMD434	M1−I230
A718NC	pMD434 + pMD418	M1−I230 + R231−end
A721N	pMD228 + pMD418	R231−end
A779C	yEPlac181 + pMD457	G156−end
A780N	pMD228 + pMD456	M1−T155
A781NC	pMD456 + pMD457	M1−T155 + G156−end
A782C	yEPlac181 + pMD459	G163−end
A783N	pMD228 + pMD458	M1−I162
A784NC	pMD458 + pMD459	M1−I162 + G163−end
A809,3 frag	pMD421 + pMD468 + pMD432	M1−T78 + P79−G273 + T274−end

[a] In the strain designations, N refers to a strain containing the amino-terminal fragment only, C refers to a strain containing the carboxyl-terminal fragment only, and NC refers to a strain coexpressing amino and carboxyl termini. WT refers to a strain expressing full-length c-*myc*-tagged cysteine-less normal receptor, and NR refers to a strain containing no *STE2* gene. [2] A methionine is the leading residue in all carboxyl-terminal fragments. This residue has been omitted from the carboxyl-terminal fragments of this table.

The plasmid pMD523 encoding a nearly full-length receptor with a deletion of residues 156−162 in the IC2 loop was created by oligonucleotide-directed mutagenesis of plasmid pMD410 with oligonucleotide ON261.

Yeast Strains and Media. The yeast strains used in this paper are summarized in Tables 1−3. The original *ste2*$^-$ host strain was A232 (*MAT***a** *ste2*-Δ*cry1*^{R} *ade2-1 his4-580 lys2*$_{oc}$ *tyr1*$_{oc}$ *SUP4-3*^{ts} *leu2 ura3 bar1-1 FUS1*::p[*FUS1*−*lacZ TRP1*]). Strain A230, used for testing dominant effects of fragments, is identical to A232 except that it contains an integrated HA epitope-tagged chromosomal *STE2*$^+$ allele (see ref *72*). Strain A952, expressing a nearly full-length receptor with a deletion in IC2, was created by transforming A232 with plasmid PMD523 containing the mutant receptor and the URA3-containing vector pMD228.

Yeast transformations were accomplished by the procedure of Gietz and Woods (*31*). Unless otherwise noted, yeast were cultured on synthetic dropout medium or YPD as described (*32*). To allow selection for G418 resistance while also maintaining *URA3* and *LEU2* markers in the expression of receptors from three fragments, cells were cultured on medium containing 1.67 mg/mL yeast nitrogen base without amino acids and ammonium sulfate (Difco), 20 mg/mL bacto-agar (Difco), 1.1 mM dextrose, 1.1 μM L-adenine, 1.0 μM L-histidine, 1.1 μM L-lysine, 1.7 μM L-tyrosine, 188.2 mM L-glutamine and 0.2 mg/mL G418 (Life Technologies).

Assays of the Yeast Pheromone Response. All assays were performed on three independent transformants. Assays of *FUS1*−*lacZ* induction and halo assays of growth arrest were performed as described previously (*19*, *24*, *33*). Briefly, β-galactosidase assays of *FUS1*−*lacZ* induction were performed with cells grown in selective media (uracil- and leucine-deficient dropout medium or G418 selective medium) to an OD_{600} of approximately 1. Cultures were then diluted to an OD_{600} of 0.04 in YPD and allowed to grow for 1−2 generations. Cells were incubated with the indicated concentrations of α-factor for 110 min. A total of 1.25×10^6 induced cells was harvested and subjected to assay of β-galactosidase activity as described (*32*). Enzymatic reactions were carried out for 60 min at 28 °C. Where the results are expressed as relative β-galactosidase activity, the activities determined for different fragment-expressing strains were normalized against the activity of A607 expressing a full-length receptor measured in the same set of assays.

Halo assays were performed by growing cells overnight to the stationary phase in selective medium as described above. A total of 4×10^7 cells was harvested and resuspended in 2 mL of water. Resuspended cells were added to 2 mL of selective medium containing 1% bacto-agar and vortexed briefly, and this mixture was poured on top of plates containing selective medium with 2% bacto-agar. This top agar was allowed to solidify for 45 min, and then the indicated amounts of α-factor in a final volume of 3 μL of water were spotted on each plate. The diameter of each halo induced by α-factor was measured after incubation of plates for 48 h at 28 °C.

Immunoblotting of α*-Factor Receptors.* Cells were cultured to an OD_{600} of 0.8−1.2. They were then frozen at −70 °C, thawed, disrupted with glass beads, prepared for electrophoresis, and transferred to nitrocellulose as described (*72*),

Table 3: Yeast Strains Expressing Noncomplementary Pairs of Receptor Fragments

strain[a]	plasmids	plasmid-encoded regions of STE2[b]
A676, N4C6	pMD431+ pMD421	M1−A198 + P79−end
A677, N3sC6	pMD428 + pMD421	M1−V152 + P79−end
A678, N3sC5	pMD428 + pMD422	M1−V152 + G115−end
A683, N4C5	pMD431 + pMD422	M1−A198 + G115−end
A684, N4C4l	pMD431 + pMD433	M1−A198 + I153−end
A688, N5lC6	pMD426 + pMD421	M1−L236 + P79−end
A689, N5lC5	pMD426 + pMD422	M1−L236 + G115−end
A690, N5lC4l	pMD426 + pMD433	M1−L236 + I153−end
A691, N5lC3	pMD426 + pMD423	M1−L236 + T199−end
A694, N6C6	pMD432 + pMD421	M1−G273 + P79−end
A695, N6C5	pMD432 + pMD422	M1−G273 + G115−end
A696, N6C4l	pMD432 + pMD433	M1−G273 + I153−end
A697, N6C3	pMD432 + pMD423	M1−G273 + T199−end
A698, N6C2s	pMD432 + pMD427	M1−G273 + G237−end
A700, N2C6	pMD430 + pMD421	M1−T114 + P79−end
A811, N5lC2l	pMD426 + pMD434	M1−L236 + R231−end
A814, N3mC4l	pMD456 + pMD433	M1−T155 + I153−end
A815, N3mC4s	pMD456 + pMD459	M1−T155 + G163−end
A816, N3lC4l	pMD458 + pMD433	M1−I162 + I153−end
A817, N3lC4m	pMD458 + pMD457	M1−I162 + G156−end
A848, N3mC5	pMD456 + pMD 422	M1−T155 + G115−end
A849, N3mC6	pMD456 + pMD 421	M1−T155 + P79−end
A850, N3lC5	pMD458 + pMD 422	M1−I162 + G115−end
A851, N3lC6	pMD458 + pMD 421	M1−I162 + P79−end
A852, N4C4m	pMD431 + pMD 457	M1−A198 + G156−end
A853, N5lC4m	pMD426 + pMD 457	M1−L236 + G156−end
A854, N6C4m	pMD432 + pMD 457	M1−G273 + G156−end
A855, N4C4s	pMD431 + pMD 459	M1−A198 + G163−end
A856, N5lC4s	pMD426 + pMD 459	M1−L236 + G163−end
A857, N6C4s	pMD432 + pMD 459	M1−G273 + G163−end

[a] In the strain designations, N*x*C*y* refers to a strain containing the amino-terminal fragment with the first *x* transmembrane segments, and the carboxyl-terminal fragment with the last *y* transmembrane segments. In cases where multiple end-points reside in the same loop, l refers to the longest fragment, m refers to the fragment with the medium length, and s refers to the shortest fragment. [b] A methionine is the leading residue in the primary translation product of all carboxyl-terminal fragments. To avoid confusion, this residue has been omitted from the designations of the carboxyl-terminal fragments of this table.

except that 20 μL of each sample was loaded on the polyacrylamide gel. Filters were blocked with 5% newborn calf serum in 10 mM sodium phosphate and 140 mM sodium chloride, pH 7.4 (PBS), for 2 h, rinsed twice with 0.05% Tween 20 in PBS, incubated with mouse anti-c-*myc* antibody (Boehringer Mannheim) at a concentration of 400 ng/mL for 2 h, rinsed five times in 5% serum in PBS, and then incubated with peroxidase-conjugated goat anti-mouse antibody (Bio-Rad Laboratories) at a dilution of 1:3000 for 2 h, washed three times with 0.05% Tween in PBS, and developed with tetramethylbenzidine as described previously (*34*).

RESULTS

Assembly of Functional Receptors from Complementary Fragments. To test for assembly and function of split receptors, the yeast *STE2* gene encoding the α-factor receptor was mutagenized to produce proteins truncated at their amino or carboxyl termini. Pairs of multicopy plasmids with different auxotrophic markers (*URA3* and *LEU2)* encoding complementary fragments of the receptor were transformed into a yeast strain containing a chromosomal deletion of the *STE2* gene. Expression from multicopy plasmids was used to provide adequate levels of expression in case receptor fragments turned out to be unstable. Truncated receptors were, with one exception (*see* Experimental Procedures), created by using oligonucleotide-directed mutagenesis to delete the unwanted portions of the gene.

The sites of receptor truncations are shown in Figure 1. Except for two amino-terminal fragments that had been previously isolated from another study, (amino acids 1−152 and 1−230), the end points were located as far away as possible from the hydrophobic transmembrane regions and adjacent to uncharged residues with small side chains. This strategy should eliminate interactions between charged amino acids and the peptide termini and place the end points of the fragments in flexible regions. In the short IC1 (first intracellular; see Figure 1) loop, between TM1 (first transmembrane segment) and TM2, the choices were limited and a site near the membrane was chosen that was between Thr78 and Pro79. Following failure to reconstitute a functional receptor from fragments split between Ile230 and Arg231 in IC3, a second site for splitting was chosen between Leu236 and Gly237. Similarly, the splits between Thr155 and Gly156 and between Ile162 and Gly163 in IC2 were chosen after failure to reconstitute function with fragments that were split between Val152 and Ile153.

The ability of the split receptors to signal in the presence of α-factor was assayed by testing for induction of the yeast pheromone response pathway. The yeast strain used as a host for the expression of split receptors contains the *E. coli lacZ* gene fused to *FUS1*, a gene that is transcriptionally induced by activation of the pheromone response pathway. Thus, function of the receptors can be qualitatively assayed on culture dishes containing both α-factor and the colorimetric β-galactosidase substrate X-gal or quantitatively assayed in liquid cultures with the β-galactosidase substrate ONPG.

Most of the yeast strains expressing complementary pairs of receptor fragments exhibited significant pheromone responses, indicating that the fragments assemble to form functional receptors (Figure 2). Among these functional split receptors are examples where the site of the split resides in each of the loops connecting transmembrane segments. The magnitudes of the pheromone responses for receptors considered to be functional ranged from 14% to 70% that of the normal receptor (measured after induction with 500 nM α-factor; Figures 2 and 3). The levels of induction expressed as levels of *FUS1−lacZ* expression in the presence of 500 nM α-factor, compared with *FUS1−lacZ* expression in the absence of ligand, ranged from 2-fold to 20-fold for the split receptors, compared with 26-fold for the cells expressing the normal receptor. No yeast strain expressing any individual truncated receptor fragment with less than seven transmembrane segments responded to α-factor in the assay for *FUS1−lacZ* induction (Figure 2). None of the truncated receptors expressed individually or in any combination exhibited any significant increase in basal expression of *FUS1−lacZ* in the absence of α-factor.

The functional split receptors were activated by levels of α-factor that were similar to or slightly greater than levels required for activation of the normal receptor (Figure 3). However, two combinations of complementary fragments, one split between residues 78−79 and one split between residues 236−237, did not show clear evidence of saturation of *FUS1−lacZ* induction at the highest concentrations of

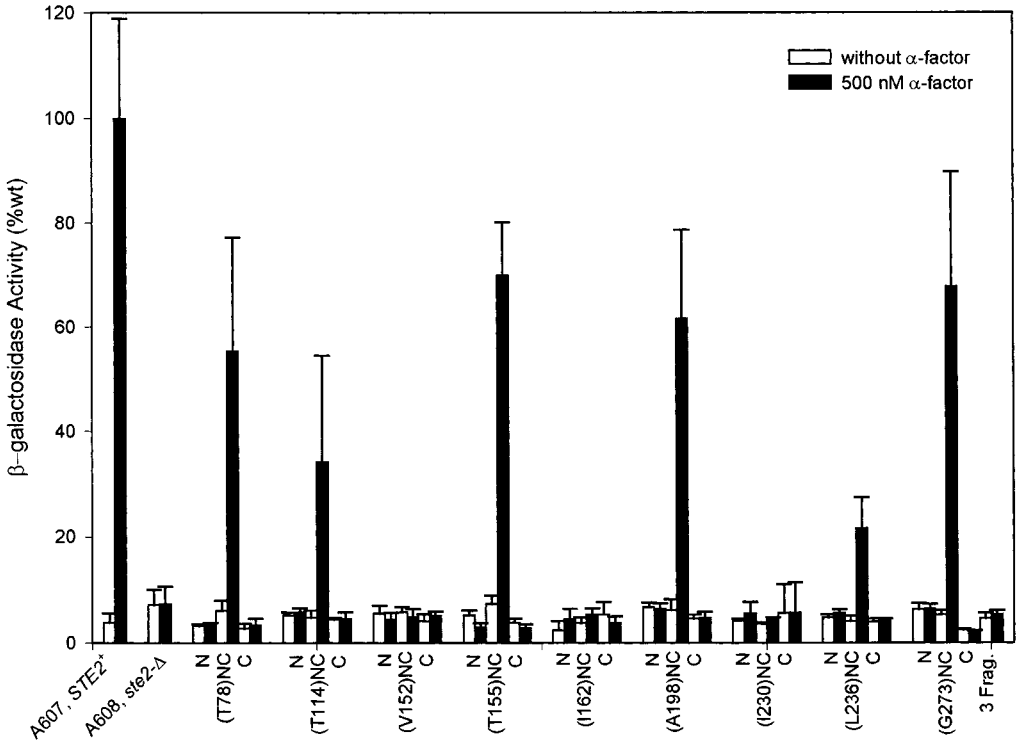

FIGURE 2: Induction of *FUS1−lacZ* expression in response to α-factor by cells expressing individual *STE2* fragments and complementary pairs of fragments. Pheromone responsiveness of various yeast strains was assayed by quantitative assays of *β*-galactosidase activity expressed from the *FUS1−lacZ* reporter gene in response to α-factor as described in Experimental Procedures. N indicates a strain expressing an amino-terminal fragment alone. C indicates a strain expressing a carboxyl-terminal fragment alone. NC indicates a strain expressing a complementary pair of amino- and carboxyl-terminal fragments. Strain A809 (designated as 3 Frag) expresses the *STE2* receptor as three complementary fragments consisting of TM1, TM7, and TM2−TM6. The indicated relative values of *β*-galactosidase activity are expressed as a percent of the activity exhibited by control strain A607 in the presence of 500 nM α-factor. At least three independent transformants were tested.

pheromone used and thus may be partially defective for α-factor binding or receptor activation.

Halo assays of growth arrest induced by various concentrations of α-factor were also conducted on split receptors (results not shown). All pairs of fragments capable of eliciting *FUS1−lacZ* induction also exhibited growth arrest in this assay, except for the pair split between Leu236 and Gly237. In this case, the low maximal level of response was apparently insufficient to surpass the threshold for growth arrest, as has been reported for other partially functional *ste2* alleles (*24*). Receptors split between residues Thr114 and Gly115 required a 2−3-fold increase in the concentration of pheromone needed to achieve a given size of halo, compared with intact receptors. The remainder of the split receptors exhibited normal sensitivities to α-factor in this assay. Receptors split after Thr78, Thr 114, and Gly273 gave rise to partially filled-in halos even at the highest α-factor concentrations, consistent with a reduced maximal response.

Not all split receptors were capable of signaling. In the IC3 loop (see Figure 1), a receptor split between Ile230 and Arg231 did not function, even though a receptor split only six residues away, between Leu236 and Gly237, was capable of moderate function. No function was detected for receptor split in the IC2 loop between Val152 and Ile153 or between Ile162 and Gly163, although a receptor split between these positions, at Thr155-Gly156, exhibited near-normal function.

As a test of a model for receptor assembly in which interactions between TM1 and TM7 direct association of the other transmembrane segments (see below), the receptor was expressed in three contiguous fragments. An amino-terminal fragment containing only TM1 was coexpressed with a fragment containing just TM7 (and the carboxyl-terminal tail) and a fragment encompassing the intervening five transmembrane segments, TM2−TM6. Cells expressing these three fragments exhibited no detectable *FUS1−lacZ* induction in response to pheromone (Figure 2).

Immunologic Detection of Receptor Fragments. To evaluate the relationship between levels of expression and function of split receptors, and as verification of the molecular weights of the truncated receptor fragments, cellular levels of carboxyl-terminal receptor fragments were assessed by immunoblotting with three tandem copies of the c-*myc* epitope inserted at the extreme carboxyl terminus of the α-factor receptor (*26*; Figures 4 and 5). Lane 1 of Figure 4A shows an immunoblot of an extract derived from cells expressing intact α-factor receptor. The heterogeneity of the predominant band above 50 kDa has previously been reported to reflect multiple glycosylation states of the protein, and the bands above 100 kDa have previously been reported to be oligomeric forms of the receptor (*35*; see below).

Cellular levels of the carboxyl-terminal receptor fragments were similar to normal levels of full-length receptor and were not strongly affected by coexpression of complementary amino-terminal fragments (Figures 4 and 5). However, coexpression of the complementary fragments led to some differences in the distributions of different bands, perhaps reflecting alterations in posttranslational modifications. In particular, fragments 199−end and 237−end showed increased densities of bands migrating more slowly than the predominant bands in cells expressing the carboxyl-terminal

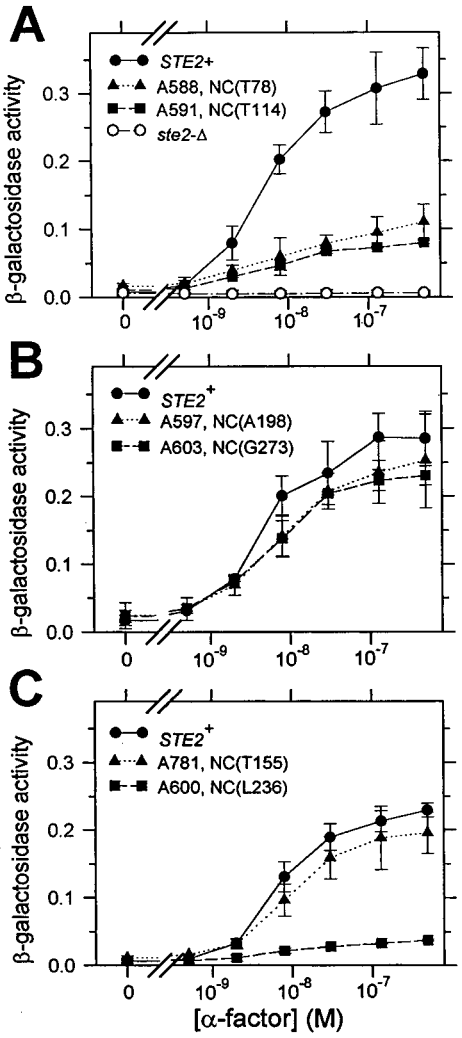

FIGURE 3: Dose−response curves of *FUS1−lacZ* induction in response to pheromone binding to functional split receptors. The pheromone responsiveness of various yeast strains expressing complementary pairs of receptor fragments was assayed by quantitative assay of β-galactosidase activity expressed by the *FUS1−lacZ* reporter gene in response to α-factor as described in Experimental Procedures. The indicated β-galactosidase activities are calculated according to Rose et al., (*32*). The legends present the strain numbers and, in parentheses, the sites of the discontinuities in the receptor. NC indicates that both amino and carboxyl termini are expressed. The three panels present results from different pairs of coexpressed fragments. In each case, the control strain, A607, was assayed in parallel with the strains being assayed.

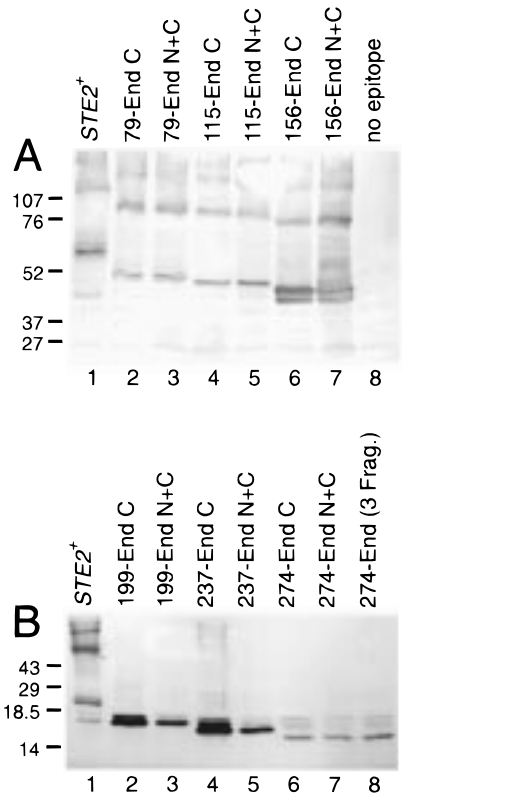

FIGURE 4: Immunologic detection of receptor fragments. Carboxyl-terminal receptor fragments and intact receptors were tagged with c-*myc* epitopes. Extracts derived from 4×10^6 cells were subjected to SDS−polyacrylamide gel electrophoresis on gels containing 9% acrylamide, transferred to nitrocellulose filters, and detected with anti-c-*myc* antibodies. In the strain designations, C refers to a strain containing the carboxyl-terminal fragment only, and N + C refers to a strain coexpressing amino and carboxyl termini. The diffuse band migrating with an apparent molecular mass of less than 60 kDa is the c-*myc*-tagged *STE2* gene product.

fragment alone (Figure 4B). This could reflect higher levels of glycosylation or altered phosphorylation of the receptor in cells that did not express the complementary fragment. The intact α-factor receptor is glycosylated at sites near the amino terminus of the protein (*35*). However, there are additional potential sites of N-glycosylation at residues Ser207 in EC2, near the amino terminus of one of the truncated fragments exhibiting extra bands, and at Ser303 in the cytoplasmic tail. Either the truncated receptors or a subpopulation of receptor fragments inserted into the membrane with incorrect transmembrane topology may undergo glycosylation at these cryptic acceptor sites when the normal amino terminus is missing. If coexpressed fragments assemble in the endoplasmic reticulum or Golgi, this may block access of glycosylating enzymes to these sites or may suppress the incorrect insertion into the membrane. In addition, phosphorylation is known to occur at sites in the carboxyl-terminal tail of the receptor (*36*). Thus, differences in phosphorylation state could also be reflected in the altered mobilities of the carboxyl-terminal fragments of the truncated receptors.

The receptor fragment encompassing residues 156−end was detected on immunoblots as two closely spaced bands of approximately equal density (Figure 4). The relative abundance of these bands was not affected by expression of the complementary amino-terminal fragment. This heterogeneity could be the result of aberrant glycosylation, multiple phosphorylated states, proteolysis of the free end of the IC2 loop, or initiation at Met165, which is located just a few residues downstream from the engineered initiation codon in this fragment.

A fraction of the intact receptors, as well as some of the larger carboxyl-terminal receptor fragments, migrated as dimers or larger aggregates on SDS gels. Oligomerization of intact receptors has been reported previously, even when samples are pretreated with high concentrations of urea prior to electrophoresis, as was the case for the samples shown in Figures 4 and 5 (*35*). It is not known whether the detection of oligomers on SDS gels is indicative of their aggregation state in cells. The failure to detect oligomerization of smaller truncated receptor fragments indicates that either (1) multiple interactions among transmembrane segments are required for

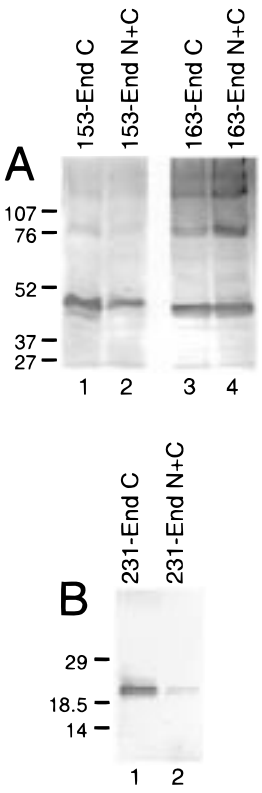

FIGURE 5: Immunologic assay of levels of expression of nonfunctional receptors. Panels A and B depict the levels of expression of the nonfunctional split receptors. Extracts from 4×10^6 cells were analyzed as described for Figure 4. Lanes are labeled as in Figure 4.

this behavior and fragments containing three or less segments do not interact strongly enough to exhibit this behavior or (2) it is the fourth transmembrane segment in particular that is responsible for oligomerization, since carboxyl-terminal fragments lacking this segment migrate exclusively as monomers on gels. Coexpression of amino- and carboxyl-terminal fragments did not lead to major changes in the pattern of oligomerization, compared with expression of carboxyl-terminal fragments alone. Thus, the detected oligomers do not appear to contain amino-terminal fragments.

Assembly of Receptors from Pairs of Fragments with Supernumerary Transmembrane Segments. To determine whether the intramolecular interactions driving assembly could accommodate the presence of extra or missing sequence elements, every possible pairwise combination of noncomplementary fragments was created by transformation of the appropriate plasmids into yeast (Table 3). No yeast strain expressing fragments comprising less than the full complement of seven transmembrane segments exhibited any significant pheromone-dependent induction of *FUS1−lacZ* expression (results not shown).

Some pairs of receptor fragments encoding supernumerary transmembrane segments exhibited high levels of *FUS1−lacZ* induction in response to α-factor (Figure 6). Three pairs of overlapping fragments display nearly normal responses to pheromone. These pairs all consisted of an amino-terminal fragment containing TM1−TM4 combined with various carboxyl-terminal fragments, each containing TM4−TM7. However, when analyzed by immunoblotting, the carboxyl-terminal fragments in these pairs all displayed multiple bands (results not shown) similar to the multiple species detected for these same TM4−TM7-containing fragments in Figures 4 and 5. Since the lower molecular weight band exhibits about the same mobility as a fragment containing only TM5−TM7, this suggests that the observed activity may actually be due to assembly of complementary fragments that have been proteolytically trimmed from the primary translation products. Because the epitope used for immunoblotting is at the extreme carboxyl terminus of the receptor, the detection of immunoreactive cleaved species indicates that trimming occurs at the amino terminus of the fragment. Proteolysis at the carboxyl terminus could remove one or two copies of the triple c-*myc* epitope (consisting of approximately 11 amino acids per repeat) while leaving partial immunoreactivity. However, the same carboxyl-terminal sequence is present in all carboxyl-terminal fragments, whereas the extent of trimming appears to depend on the site of truncation at the amino-terminal end of the fragments.

Cells expressing five additional overlapping pairs of receptor fragments (strains A691, A697, A683, A848, and A850, all containing 1−2 extra transmembrane segments; see Figure 6) exhibited 10−15% of the normal pheromone response. Although low-efficiency assembly of receptors from these fragments may be occurring, and although no obvious proteolysis is detected by immunoblotting of the carboxyl-terminal fragments expressed in these strains, there remains a possibility that the detected pheromone responses are due to proteolytic trimming of the overlapping regions from a small proportion of the amino- or carboxyl-terminal fragments. In addition, although no full-length receptor is detected by immunoblotting of these strains, it is also possible that signaling might be due to the presence of a small amount of intact receptor resulting from recombination between overlapping DNA segments (see Experimental Procedures). The presence of as little as 5% of the normal level of wild-type receptors is sufficient to provide a nearly normal pheromone response (*37*). None of the strains expressing overlapping fragments exhibited significant elevation of basal levels of *FUS1−lacZ* expression in the absence of α-factor (results not shown).

Assembly of a Functional Receptor with a Deletion in the Second Intracellular Loop. The creation of receptor fragments truncated at more than one site within the IC2 and IC3 loops made it possible to combine noncomplementary amino- and carboxyl-terminal fragments to create split receptors containing deletions in these loop regions. Surprisingly, the pair of fragments containing a deletion of residues 156−162 in IC2 exhibited a partial pheromone response (Figure 7). The same deletion introduced into an intact receptor, not expressed from fragments, yielded a response to α-factor that was indistinguishable from that of the normal full-length *STE2*$^+$ allele. No other pairs of fragments lacking regions of the receptor sequence, including a slightly larger deletion encompassing residues 153−162 in IC2, an adjacent deletion of residues 153−155, or a deletion of residues 231−236 in IC3, could combine to produce receptors with any detectable function (results not shown).

Truncated Receptor Fragments Do Not Interfere with the Function of Coexpressed Normal α-Factor Receptors. While the mechanism of signaling by GPCRs, as we currently understand it, does not involve oligomerization of the receptor, there are several reports that receptors may exist as oligomers (*38*−*42*). Some of these are based on the ability

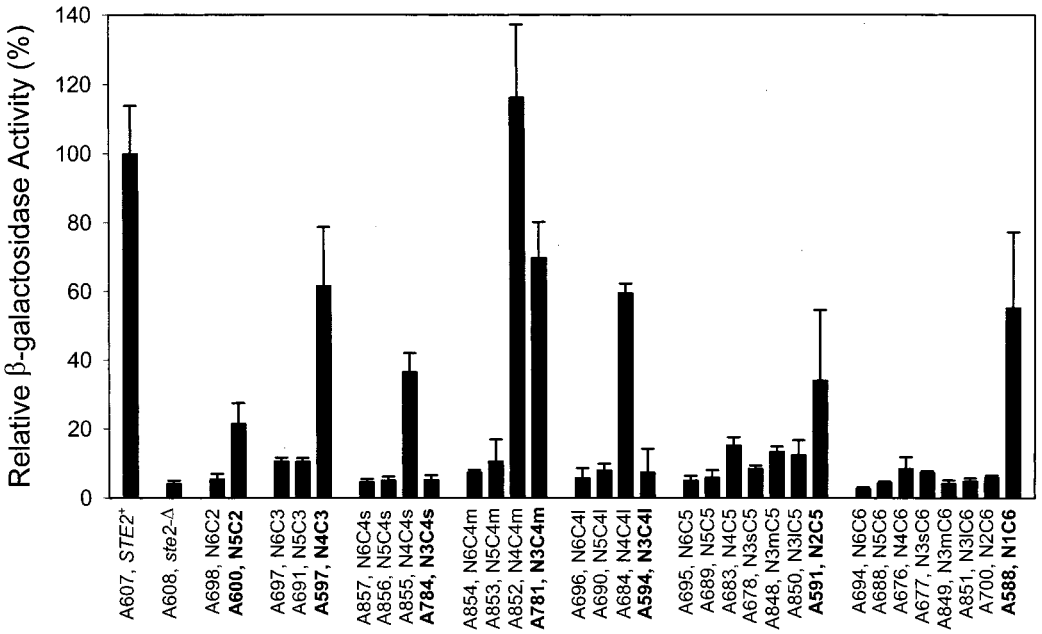

FIGURE 6: Pheromone response of receptors assembled from fragments containing supernumerary transmembrane segments and compared with receptors assembled from complementary fragments. Strains are labeled N*x*C*y*, where N refers to an amino-terminal fragment with the first *x* transmembrane segments, and C refers to a carboxyl-terminal fragment with the last *y* transmembrane segments. The designations s, m, and l refer to the small, medium, and large fragments encompassing the indicated number of predicted transmembrane segments. Strains with complementary amino- and carboxyl-terminal fragments are shown in boldface type. The receptor pairs have been grouped with respect to the particular carboxyl-terminal fragments expressed. The yeast strains are listed in Table 3. Relative β-galactosidase activity is calculated as described for Figure 2. The data presented for each strain are derived from 3−6 assays of at least three independent transformants.

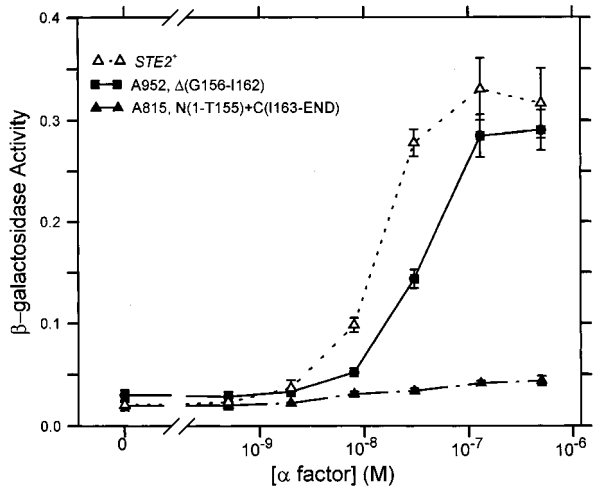

FIGURE 7: Dose−response curves for split and intact receptors containing a deletion of amino acid residues 156−162. *FUS1*−*lacZ* induction was assayed as for Figure 3.

of synthetic peptides corresponding to transmembrane segments of GPCRs to interfere with signaling or to alter the pharmacological properties of receptors (*39*). This interference, which has not yet been tested in the yeast system, has been attributed to a competition in which the synthetic peptides prevent functionally important oligomerization of receptors. However, the concentrations of peptides used in these studies are generally quite high and the effects could be the result of competition for intramolecular interactions among transmembrane segments that are important for receptor folding or function. If introduction of synthetic peptides interferes with signaling by preventing receptor oligomerization, coexpression of truncated and full-length receptors could lead to similar interference, since truncated receptor fragments encompassing one or more transmembrane segments would be expected to exhibit affinities for intact receptors that are at least as strong as those of synthetic peptides corresponding to single transmembrane segments. Furthermore, dominant negative effects of mutant receptors have recently been detected in yeast (*72*).

To test for dominant interference of truncated receptor fragments with signaling by normal receptors, the truncated fragments were expressed in cells that also contained a normal receptor. The likelihood of detecting such interference was enhanced by expressing the fragments from multicopy plasmids in a strain where the *STE2*$^+$ allele was present as a single integrated chromosomal copy. None of the strains expressing the truncated receptors displayed any significant defect in induction of *FUS1*−*lacZ* expression compared with a strain expressing *STE2*$^+$ alone (Figure 8).

CONCLUSIONS

No Single Covalent Connection between Transmembrane Segments of the Yeast α*-Factor Receptor Is Required for Receptor Folding or Function.* We have created a series of amino- and carboxyl-terminal receptor fragments truncated in each of the loops connecting transmembrane segments. No receptor fragment lacking any transmembrane segment exhibited any signaling function, consistent with the results of previous deletional and insertional mutagenesis studies (*43*, *44*). However, coexpression of a number of pairs of complementary receptor fragments led to recovery of pheromone-responsive signaling, indicative of intragenic allelic complementation. Intragenic complementation of alleles of *STE2* had previously been observed in several cases involving coexpression of a point mutant with insertional mutants (*44*). In the present study, the pairs of complementary

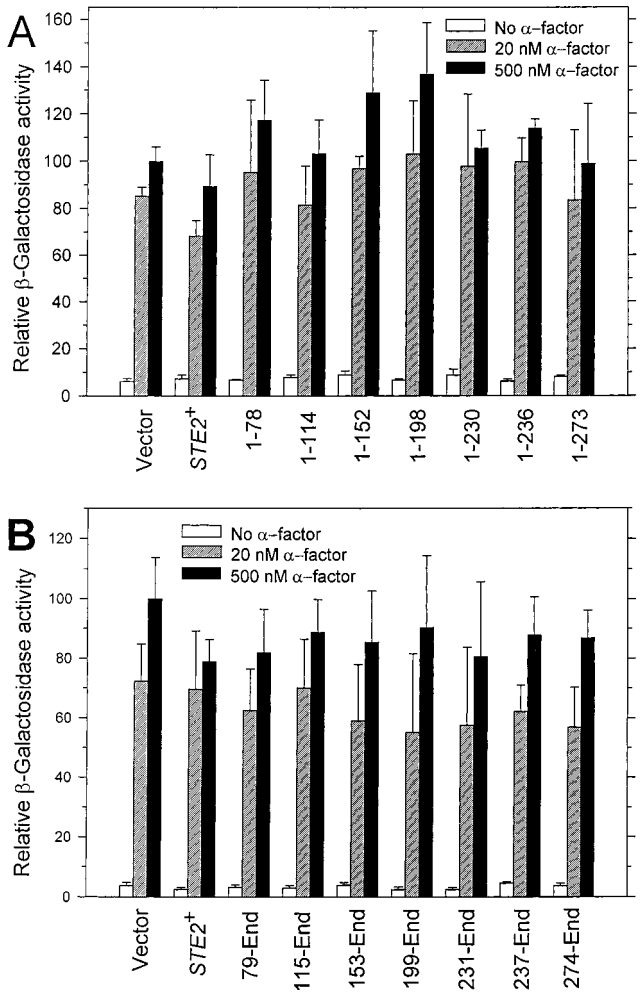

FIGURE 8: Pheromone response of normal α-factor receptors coexpressed with an excess of truncated receptor fragments. The indicated STE2 alleles were expressed from multicopy plasmids in yeast strain A230 containing an integrated chromosomal copy of *STE2*$^+$. (A) Amino-terminal fragments; (B) carboxyl-terminal fragments. The particular strains used for the assays are shown in Table 1.

fragments capable of coassembling into functional receptors include examples with discontinuities in each of the intracellular and extracellular loops connecting transmembrane segments. This demonstrates that there is no absolute requirement for covalent connection of any two transmembrane segments or connecting loops for correct folding or assembly of this protein.

Assembly of coexpressed receptor fragments appears to result from interactions among transmembrane segments, consistent with a two-stage model of membrane protein folding in which hydrophobic segments are inserted into the membrane as independent α-helices and then assembled into tertiary structures (*4*). Interactions between the short aqueous-facing ends of receptor fragments would not be expected to provide sufficient affinity or specificity to drive receptor assembly, particularly in the presence of nonnative amino- and carboxyl-terminal groups at the site of the split. This means that the structure of the transmembrane region of the α-factor receptor must be dictated by some combination of interactions between transmembrane segments and connecting loops. However, two of the fragments that coassemble with their complementary counterparts (fragments M1−T78 and T274−end) contain no intact loops. In these cases, assembly must be driven by interactions among transmembrane segments or between loops and the amino- or carboxyl-terminal tails. The carboxyl-terminal tail is dispensable for receptor function and is thus unlikely to play a critical role in folding. Furthermore, we have been unable to detect an interaction between the carboxyl-terminal tail and a truncated fragment containing seven transmembrane segments without the tail (C. M. Sommers, N. P. Martin, and M. E. Dumont, unpublished results).

In principle, assembly of some pairs of coexpressed fragments could be occurring through interactions of each fragment with a third protein species, such as the trimeric G protein, rather than through direct interactions among transmembrane segments. However, since the G protein is not thought to be embedded in the membrane, such tertiary interactions would have to involve the intracellular aqueous regions of the receptor. The fact that some fragments contain no loops makes this possibility unlikely as a general mechanism of assembly.

The variety of receptor fragment pairs that assemble correctly and produce a functional receptor can be explained by two different models for the interaction among transmembrane segments. In the first model, assembly would be driven by multiple overlapping interactions among transmembrane segments. For example, association of a fragment containing only the first segment with its coexpressed complementary piece would result from interactions of the first transmembrane segment with various other segments including the second transmembrane segment. However, the association of a fragment containing both the first and second transmembrane segments with its complementary piece cannot be driven by any interaction between the first and second segments and must, instead, be driven by other interactions among transmembrane segments. A second, simpler, but less likely, model is that all the associations between complementary receptor fragments are driven by an affinity between the first and seventh transmembrane segments, since each of the amino-terminal fragments we tested contains the first transmembrane segment and each of the carboxyl-terminal fragments contains the seventh transmembrane segment. This model predicts that severing the loops that connect the first and seventh transmembrane segments to the rest of the molecule should prevent assembly, which is consistent with the observed failure of assembly of three separate fragments comprising of the first transmembrane segment TM1, the seventh transmembrane segment TM7, and the intervening five segments TM2−TM6. However, an alternative explanation for failure of these three fragments to assemble is that the fragment containing TM2−TM6 is unstable or is not efficiently expressed. (The results obtained with pairwise combinations of fragments demonstrate that pieces containing just TM1 or just TM7 can be synthesized and incorporated into functional receptors.) It has previously been shown that rhodopsin, another example of a G protein-coupled receptor, and bacteriorhodopsin, a proton pump composed of seven transmembrane segments, can be assembled from three individual fragments (*45*−*47*). However, TM1 and TM7 were not expressed as individual fragments in these studies.

In previous studies of the assembly of G protein-coupled receptors from coexpressed fragments, only certain pairs of fragments that were split between particular transmembrane

segments were capable of associating to reconstitute ligand binding sites, and only a subset of the split receptors that bound ligand were capable of actually transducing a signal (*9*−*11*, *22*). Similarly, in studies of other transmembrane proteins, only pairs of fragments split between certain transmembrane segments can reconstitute function (*7*, *12*−*14*). The robustness of the α-factor receptor to fragmentation raises the possibility that the ability to assemble from pairs of complementary fragments cleaved at any loop between transmembrane segments might be a general property of G protein-coupled receptors and other multispan transmembrane proteins. Failure to recover function from certain combinations of fragments would then be explained by deleterious effects of the particular choice of sites at which the discontinuity was introduced, implying that fine-tuning of these sites may allow functional assembly of pairs of fragments of membrane proteins that have previously been reported to be incapable of associating.

One component of the deleterious effects of particular sites of splitting of α-factor receptors may be the introduction of an initiator methionine at the site of the discontinuity. All of the carboxyl-terminal fragments of the α-factor receptor that could be successfully reconstituted with complementary amino-terminal fragments contain glycine, threonine, or proline as the penultimate amino acid at the amino-terminal end. Presence of these residues at this penultimate position leads to at least partial cleavage of the initiator methionine (*29*). The three carboxyl-terminal truncations that could not be successfully assembled into receptors contain arginine and isoleucine at the penultimate positions, which usually does not allow amino-terminal methionine cleavage (*29*). However, the physical basis for such an effect of methionine cleavage is not clear, since receptor truncation also introduces peptide amino- and carboxyl-terminal groups that would be expected to have significant effects on receptor structure and function.

In some previous studies of split membrane proteins, fragments did not accumulate in cells unless both complementary pieces of the protein were expressed (*48*). In other cases, individual fragments could be stably expressed (*10*, *49*, *50*). On the basis of the behavior of the fragments of the α-factor receptor that we have been able to detect by immunoblotting, this receptor falls clearly into the latter class. This may, in part, explain the remarkable robustness of the α-factor receptor to the introduction of discontinuities. The stability of individually expressed receptor fragments in cells means that association between fragments could be occurring during biosynthesis or targeting of the receptor fragments to the plasma membrane, following insertion of the fragments into the plasma membrane, or during endocytosis and recycling of receptors. Cellular levels of two carboxyl-terminal fragments (Ile153−end and Arg231−end) were lower when expressed in the presence of the complementary fragment than they were when expressed singly (Figure 5). This raises the possibility that partially or incorrectly assembled receptors can be degraded by a proteolytic mechanism that does not recognize the individually expressed fragments.

Activation of the pheromone response pathway does not depend strongly on the number of receptors expressed on the cell surface (*37*). Thus, the ability to detect the pheromone response initiated by receptors assembled from coexpressed fragments does not necessarily mean that all the fragments present in the cell associate to form functional receptors. In fact, the number of different combinations of truncated fragments of the α-factor receptor that are capable of coassembling into functional receptors may reflect the sensitivity of the assay for *FUS1*−*lacZ* induction in detecting small numbers of functional receptors. On the other hand, the sensitivity of the response suggests that those combinations of fragments that are not capable of mounting a pheromone response either assemble into fully functional receptors at yields of less than a few percent or assemble with higher yields into at least partially defective receptors.

The ability of coexpressed fragments to assemble into functional receptors implies that carboxyl-terminal fragments are capable of inserting into the plasma membrane with the correct topological orientation. Sequences at the amino termini of proteins that traverse the membrane multiple times appear to determine the proteins' overall topological orientations by specifying the orientation of the first transmembrane segment. The remaining carboxyl-terminal transmembrane segments must then insert in an alternating pattern. Like most G protein-coupled receptors, the α-factor receptor does not contain a cleaved targeting sequence; therefore insertion of both normal receptors and truncated receptor fragments must be dictated by the sequences in the mature protein. The ability of carboxyl-terminal fragments of the α-factor receptor to insert correctly into the membrane in the absence of information from the normal amino terminus could be explained in two ways: (1) The fragments could coassemble with amino-terminal fragments at an early stage of biosynthesis, thus inserting into the membrane in a manner similar to that followed by the intact receptor. Correct insertion of other membrane proteins may be capable of being directed by sequences expressed in trans (*51*). (2) Sequence information at the amino termini of the truncated fragments could be acting to correctly insert the transmembrane segments. For example, Hartmann et al. (*52*) identified an algorithm for quantitating the difference in charge between regions on the cytoplasmic and extracellular faces of transmembrane segments that correlates well with the topology of insertion. This algorithm correctly predicts the transmembrane orientation of TM1 of the normal α-factor receptor and a series of mutated receptors (*53*). In considering carboxyl-terminal fragments of the α-factor receptor, there is a correlation between the charge difference calculated with this algorithm and the topological orientations of fragments that have TM2 through TM7 as their amino termini (see Table 1). Each transmembrane segment that has its amino-terminal end facing the cytoplasm in the intact receptor has a charge difference Δ(C−N) of greater than or equal to zero. Each segment with the opposite orientation has a Δ(C−N) less than or equal to zero. Failure of some fragments to assemble into functional receptors does not appear to result from incorrect topological orientation in the membrane, since the nonfunctional fragment pairs have Δ(C−N) values that are at least as favorable for correct insertion as the values for fragments that are capable of assembling into functional receptors.

Expression of Noncomplementary Receptor Fragments. We have tested for assembly of all possible noncomplementary pairs of the truncated amino- and carboxyl-terminal receptor fragments described above. Although near-normal

and partially defective pheromone responses were detected in a number of strains expressing pairs of fragments containing one or two supernumerary transmembrane segments, signaling by these strains can be at least partially explained by proteolytic removal of overlapping sequences. Thus, while it remains possible that certain combinations of overlapping receptor fragments may be capable of assembling into partially functional receptors, as has been seen with some other transmembrane proteins (*54*−*56*), the structure of the α-factor receptor is not, in general, sufficiently plastic to accommodate the presence of extra transmembrane segments.

Most of the Second Intracellular Loop Is Not Required for Receptor Function. Receptors containing a deletion of seven amino acids (residues 156−162), comprising most of the hydrophilic IC2 loop, exhibit normal function. In contrast to this result, IC2 has been identified as a critical site of interaction with G protein for certain mammalian receptors (*57*, *58*) and as a contributing factor for many others (*59*−*61*). In the α-factor receptor, sensitivity of the third intracellular loop to mutagenesis has previously been presented as evidence that, as is the case for many mammalian receptors, it is the IC3 loop that interacts directly with the G protein (*62*, *63*).

Failure of Truncated Receptors to Exhibit Dominant Negative Effects. The failure to detect dominant negative effects of truncated α-factor receptors shown in Figure 8 stands in contrast to the previous detection of dominant effects of certain receptors in the pheromone response pathway of yeast. Dominant negative receptors containing point mutations have recently been identified in genetic screens and appear to be fairly common (*72*). In addition, expression of normal α-factor receptors in the same strains as hypersensitive or constitutively active mutant *STE2* alleles suppresses the effects of these mutant alleles in a dominant fashion (*64*−*67*). Such dominant effects could be mediated by receptor oligomerization or by preferential association of a critical component of the signaling pathway, such as the G protein, with the dominant receptor alleles. If receptor oligomerization plays an important role in signaling that can be inhibited by introduction of competing transmembrane segments in the form of synthetic peptides, then truncated receptors containing as many as six transmembrane segments would, if present at adequate levels, be expected to interfere with signaling by normal receptors. Receptors expressed from multicopy plasmids are present at levels 15−20-fold higher than the same receptors expressed from chromosomal loci (*72*). Since immunoblotting analyses indicate that truncated receptors are present in cells at about the same levels as intact receptors expressed from the same vectors (Figure 4), the strains used for testing for dominant effects of truncated receptors appear to contain a 15−20-fold excess of truncated over normal receptors. This is similar to the level of expression that previously allowed detection of dominant negative effects (*72*). However, it is possible that inhibitory effects might be seen if truncated receptors could be expressed at higher levels. Thus, the lack of dominant inhibitory effects by truncated receptors indicates either that receptor oligomerization is not important for signaling or that the affinity of every truncated receptor for binding to normal receptors is much lower than the affinity of normal receptors for each other.

If the dominant receptor interactions in yeast are actually caused by sequestration of G protein, rather than oligomerization, truncated receptors containing certain cytoplasmic-facing regions important for docking with G protein might be expected to competitively interfere with G protein activation by coexpressed normal receptors. Under this scenario, the failure to observe such effects indicates that no truncated receptor retains enough native structure to allow it to competitively inhibit the interaction of normal receptors with G proteins.

Applications of Split Receptors. Intramolecular disulfide cross-linking of cysteine residues introduced by site-directed mutagenesis has the potential to provide detailed maps of the structures of transmembrane regions of proteins (*23*). However, detection of cross-links in G protein-coupled receptors is hindered by the failure of intramolecular disulfides to change the mobility of these proteins on SDS−polyacrylamide gels (*68*). This problem can be circumvented by proteolytic digestion of membrane proteins so that the cross-links that are formed connect two small fragments. Such cross-linked species generally exhibit greatly altered electrophoretic mobilities compared to those of the individual proteolytic fragments. However, problems with this approach include the difficulty of finding specific sites of proteolysis positioned so as to detect a wide variety of cross-links, the difficulty of obtaining reproducible and quantitative proteolysis of proteins in crude cell extracts, and the difficulty of conducting proteolysis of plasma membrane proteins in organisms such as yeast, where cell walls can block access to the membrane. Expression of membrane protein fragments from separate genes provides an avenue for overcoming these difficulties that also allows rapid testing of many combinations of fragments containing cysteine residues introduced at different positions by site-directed mutagenesis (*22*, *23*).

The diversity of interactions detected between α-factor receptor fragments reinforces the previously proposed idea that coexpression of fragments could provide a way of modulating the function of defective G protein-coupled receptors or other membrane proteins. For example, expression of carboxyl-terminal fragments of the V2 vasopressin receptor partially restores function to coexpressed mutant vasopressin receptors known to be responsible for nephrogenic diabetes insipidus (*69*). Similarly, coexpression of an overlapping fragment of the luteinizing hormone receptor with a truncated version of this receptor associated with Leydig cell hypoplasia could partially restore function to the truncated version (*56*). There are a number of conditions affecting human disease in which mutations lead to the production of truncated or mutated G protein-coupled receptors (*70*, *71*). The promiscuity with which various fragments of the α-factor receptor can coassemble indicates that it may be generally possible to identify a receptor fragment that can be coexpressed with truncated or mutated receptors to form a complex with restored or altered signaling capabilities.

ACKNOWLEDGMENT

We thank Elizabeth Grayhack for valuable suggestions regarding these experiments. In addition, we are grateful to Eric Phizicky for helpful discussions and to Karen Kim and Elizabeth Grayhack for their comments on the manuscript.

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BI982062W