Tyr266 in the Sixth Transmembrane Domain of the Yeast α-Factor Receptor Plays Key Roles in Receptor Activation and Ligand Specificity†

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ABSTRACT: To identify interactions between Ste2p, a G protein-coupled receptor of the yeast Saccharomyces cerevisiae, and its tridecapeptide ligand, α-factor (WHWLQLKPGQPMY), a variety of α-factor analogues were used in conjunction with site-directed mutagenesis of a targeted portion of Ste2p transmembrane domain six. Alanine substitution of residues in the 262−270 region of Ste2p did not affect pheromone binding or signal transduction, except for the Y266A mutant, which did not transduce signal yet exhibited only a small decrease in α-factor binding affinity. Substitutions with Ser, Leu, or Lys at Y266 also generated signaling-defective receptors. In contrast, Phe or Trp substitution at Y266 retained receptor function, suggesting that aromaticity at this position was critical. When coexpressed with WT receptor, the Y266A receptor exhibited a strong dominant-negative phenotype, indicating that this mutant bound G protein. A partial tryptic digest revealed that, in the presence of agonist, a different digestion profile for Y266A receptor was generated in comparison to that for WT receptor. The difference in trypsin-sensitive sites and their negative dominance indicated that the Y266A receptor was not able to switch into an "activated" conformation upon ligand binding. In comparison to WT Ste2p, the mutantY266A receptor showed increased binding affinity for N-terminal, alanine-substituted α-factor analogues (residues 1−4) and the antagonist [desW[1],desH[2]]α-factor. A substantial decrease in affinity was observed for α-factor analogues with Ala substitutions from residues 5−13. The results suggest that Y266 is part of the binding pocket that recognizes the N-terminal portion of α-factor and is involved in the transformation of Ste2p into an activated state upon agonist binding.

Receptors in the cell membrane coupled to heterotrimeric GTP-binding proteins (G protein)[1] control cellular responses to a variety of stimuli including hormones, neurotransmitters, light, and odorants (*1*, *2*). Within the human genome, there are hundreds of different G protein-coupled receptors (GPCRs) (*3*, *4*). Although differing in primary amino acid sequence, all GPCRs have the same overall topology: seven transmembrane domains connected by intracellular and extracellular loops, an extracellar N-terminus, and an intracellular C-terminus (*5*, *6*).

Ste2p of the yeast *Saccharomyces cerevisiae* has been used frequently as a model for studying the structure and function of GPCRs (*7*, *8*). This receptor recognizes the tridecapeptide α-factor pheromone (WHWLQLKPGQPMY). Binding of α-factor to Ste2p activates a G protein-mediated signaling cascade that is highly conserved within eukaryotic signaling pathways. In addition to using Ste2p as a model GPCR, α-factor itself has been studied extensively as a representative GPCR peptide ligand. Covalently constrained analogues (*9*), peptidomimetic analogues (*10*), analogues with residue substitutions (*11*, *12*) including Ala-scanning (*13*), and antagonists (*14*) have been synthesized and studied with regard to their receptor binding, ability to initiate signal transduction, and conformation. These studies have revealed residues or segments of α-factor with dominant roles in forming a β-turn in the middle of the bound pheromone (residues 7−10), in binding to the receptor (residues 10−13), and in stabilizing the active conformation of the receptor to initiate signal transduction (residues 1−4).

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[1] Abbreviations: G protein, heterotrimeric GTP-binding protein; GPCR, G protein-coupled receptor; Ste2p, α-factor receptor encoded by the *STE2* gene; TM, transmembrane domain; EL, extracellular loop; WT, wild type; Nle, norleucine; [^{3}H]α-factor, [^{3}H-Pro8,Nle^{12}]α-factor; Bpa, benzoylphenylalanine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; MOPS, 3-morpholinopropanesulfonic acid; TPCK, tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Orn, ornithine; standard one-letter abbreviations for amino acids are used.

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Recently we initiated studies to define specific residue-to-residue interactions between α-factor and Ste2p. We reported that Q10 of α-factor closely interacted with S47 and T48 of Ste2p at the junction between TM1 (transmembrane one) and the N-terminal tail (*15*), and photoaffinity labeling (*16*) showed that the N-terminus of α-factor interacted with a region of Ste2p encompassing TM6−EL3−TM7 (transmembrane domain six−extracellular loop three−transmembrane domain seven). On the basis of these recent results, we proposed a model in which the C-terminus of α-factor is positioned near TM1, while the N-terminus of α-factor binds in a pocket composed of TM3, TM5, and TM6 (*15*).

A number of studies with GPCRs, including Ste2p, have demonstrated the involvement of TM5 and TM6 in receptor activation. In the rhodopsin-like family of G protein-coupled receptors TM6 has been shown to play a major role in ligand binding and in producing the resulting functional response (*17*). In Ste2p, analysis of constitutively active mutants indicated that residues in the sixth transmembrane domain play a key role in receptor activation (*18*, *19*). TM5 and TM6 of Ste2p have been implicated as important for receptor activation because the third intracellular loop, flanked by TM5 and TM6, interacts with the Gαprotein (*20*, *21*). Extensive screens to identify dominant-negative mutations in the receptor revealed that most mutants exhibiting this phenotype were mapped to the extracellular ends of the transmembrane domains, especially in TM5 and TM6 (*22*−*24*). Prior work revealed that mutation Y266C in TM6 of Ste2p resulted in a receptor that retained significant binding affinity but was defective in signaling (*22*). Furthermore, the Y266C receptor exhibited a strong dominant-negative phenotype when coexpressed with WT; this phenotype could be reversed by concomitant overexpression of the G protein subunits. This same dominant-negative receptor had also been identified as a second-site suppressor of the mutant E143K in TM3 of Ste2p (*25*), suggesting that the presence of Y266C results in alterations in the packing of the transmembrane domains, resulting in suppression.

To more fully test our hypothesis that the extracellular portion of TM6 of Ste2p interacts with the N-terminus of α-factor, we carried out the present study. We describe the effects resulting from mutation of nine residues in a portion of TM6 adjoining EL3 adjacent to the membrane extracellular interface. Focusing on the special properties of the Y266A mutation, we carried out detailed binding and biological studies on these mutated receptors using a series of Ala-scanned α-factor analogues previously synthesized in our laboratory. Our results indicate that Y266 is a strategic residue critical for receptor function and for the recognition of the N-terminus of α-factor.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. The strain LM102 (*26*) was used for receptor binding and growth arrest assay. The relevant genotype is *MAT**a**, bar1 leu2 ura3 FUS1-lacZ::URA3 ste2-dl* (α-factor receptor coding region deletion). LM102 was used as the recipient for plasmids encoding WT and site-directed mutants of *STE2*. Constructs expressed in this strain background were then used to measure pheromone-induced growth arrest (halo assay) and to determine pheromone binding. The strain carried the *bar1* mutant allele inactivating the Bar1p protease that is responsible for degradation of α-factor. The *CEN*-based yeast/bacterial shuttle vector, pGA314.WT (*27*), containing a *TRP1* selectable marker and WT *STE2* under the control of its native promoter was used as a template for the site-directedmutagenesis of the receptor. This plasmid contains a strong terminator sequence, resulting in high transcription efficiency and overexpression of Ste2p. To test for receptor mutants that exhibit a dominant-negative phenotype, pGA314 plasmids encoding either WT or site-directed mutations in *STE2* were transformed into the strain LM23-3az, a parental strain of LM102, that has a genomic copy of the WT *STE2* gene in its native chromosomal locus (*26*). Additionally, the dominant-negative phenotype was assessed in the presence of the high-copy (2 μm) plasmid pMD82, encoding the G protein α, β, and γ subunits (*GPA1*, *STE4*, and *STE18*) under the control of their native promoters (*22*). Since pMD82 and the pGA314-based plasmids contained different selectable markers (*LEU2* and *TRP1*, respectively) both could be maintained simultaneously in LM23-3az. For the limited trypsin digest experiments, the strain BJS2-1 was generated from the protease-deficient strain BJ2168 (*28*) by disrupting the chromosomal copy of *STE2* with the kanamycin resistance cassette as described by Güldener et al.(*29*). The relevant genotype of BJS2-1 is *MAT**a** prc1−407 prb1−1122 pep4−3 leu2 trp1 ura3−52 ste2::kan*^{r}. Limited trypsin digests were also completed in a diploid host strain, CG990 (*MAT**a**/α ura3−52 lys2−801 trp1-Δ1 ade2−101*) This strain was transformed with the plasmid pNED (*30*), a high-copy-number plasmid encoding epitope-tagged *STE2* under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. PCR primers were designed to amplify a 400 bp region from pGA314 containing the Y266A mutation. The mutation was engineered into pNED by in vivo ligation (*31*) and transformed into CG990.

Site-Directed Mutagenesis. Single-stranded phagemid DNA of pGA314.WT was prepared by infecting *E. coli* strain CJ236 (*ung*$^-$,*dut*$^-$) carrying pGA314.WT with the helper phage M13KO7 (*32*). Oligonucleotide-directed mutagenesis of single-stranded phagemid DNA was completed as described by Kunkel et al. (*33*). The product of the mutagenesis reaction mixture was transformed into *E. coli* strain DH5α (Invitrogen, Carlsbad, CA), and transformants were selected on medium containing ampicillin. Plasmids were then isolated from transformants using the Wizard Miniprep kit (Promega, Madison, WI). After sequence confirmation, constructs were transformed into yeast strain LM102 (*ste2*-deletion strain), and transformants were selected by their growth in the media lacking tryptophan. All primers were purchased from Sigma/Genosys (The Woodlands, TX). DNA sequencing was carried out in the Molecular Biology Resource Facility located on the campus of the University of Tennessee.

Peptides Used in This Study. L-Norleucine, which is isosteric with L-methionine, was incorporated at position 12 to replace L-methionine in all of the analogues. The insertion of Nle in place of methionine improved the synthesis and the stability of the resulting peptide. This replacement to form [Nle^{12}]α-factor was previously shown to result in an analogue with activity and binding affinity equal to those of the native pheromone (*34*). Syntheses of α-factor and its analogues used

in this study were described previously (*13*, *14*). The structures of all peptides were verified by amino acid analysis and mass spectrometry. For [^{3}H]α-factor, synthetic [Nle^{12}]α-factor was labeled by reduction of dehydroproline-containing α-factor by the TR3 hydrogenation procedure of Amersham Biosciences (Piscataway, NJ) and purified as described previously (*34*). The photoaffinity labeling analogue Bpa^{1}Y^3-(^{125}I)R^{7}F^{13} α-factor was prepared as described previously (*16*).

Growth Arrest (Halo) Assay. Yeast nitrogen base medium with ammonium sulfate without amino acids (Difco, Kansas City, MO) with 2% glucose (SD medium), supplemented with histidine (20 μg/mL), leucine (30 μg/mL), and methionine (20 μg/mL), was overlaid with 4 mL of cell suspension (2.5×10^5 cells/mL, 1.1% Nobel agar). Filter disks (sterile blanks from Difco), 6 mm in diameter, were impregnated with 10 μL portions of peptide solutions at various concentrations and placed onto the overlay. The plates were incubated at 30 °C for 24−36 h and then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo including the diameter of the disk. All assays were completed at least three times, with no more than a 2 mm variation in halo size for a particular amount of each peptide. The data were plotted as halo size versus the amount of peptide, and linearized by regression analysis using Prism software (GraphPad, San Diego, CA). To compare the relative activities of α-factor with different receptors, the amount of peptide producing a halo of a particular size was determined from the regression line of dose−response curves.

Binding Assays. Saturation and competition binding assays were performed using tritiated α-factor as previously described (*15*). Each experiment was repeated at least three times, and each data point measured in triplicate. Data curves were fit using single-site competition, nonlinear regression analysis software (GraphPad Prism). K_D and B_{max} values were determined from saturation binding assays for WT and all receptors mutated at Y266. K_i values with WT and Y266A receptor for a series of α-factor analogues were calculated using the equation of Cheng and Prusoff (*35*), where $K_i = EC_{50}/(1 + [\text{ligand}]/K_D)$. For the Ala-scanned series of receptors (F262A, I263A, L264A, A265G, S267A, L268A, K269A, and P270A) where K_D values were not determined, IC_{50} values were reported as determined directly by nonlinear regression of the competition binding data.

Trypsin Digest. Plasmid pGA314 encoding WT or Y266A mutant *STE2* was transformed into strain BJS2-1. In a parallel set of experiments, plasmid pNED encoding WT or Y266A mutant *STE2* was transformed into the diploid strain CG990. Cells were grown in MLT medium (*36*) without tryptophan to maintain selection for the plasmid. Membranes were prepared from mid-log-phase cells. All steps were performed at 4 °C. The cells were lysed with glass beads in buffer (50 mM HEPES, pH 7.5, 5 mM EDTA) supplemented with protease inhibitors (1.5 μg/mL PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin A) using a cell homogenizer (B. Braun Instruments, Allentown, PA). Unbroken cells, cell wall debris, and glass beads were removed by centrifugation (700*g*, 30 min) and the membranes collected by centrifuging the supernatant (100000*g*, 60 min). The resulting membrane pellet was resuspended in buffer (50 mM HEPES, pH 7.5, 20% glycerol) plus protease inhibitors and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The membranes were stored at −80 °C until use.

Membranes (80 μg per sample) were harvested, washed, and resuspended in YM1-i (*27*) and incubated with either α-factor or the antagonist [desW^{1}desH^2]α-factor at a final concentration of 5 μM for 30 min at room temperature. A control with no pheromone supplement was run in parallel. Tryptic digestion was performed essentially as previously described (*37*). Upon completion of the incubation interval, membranes were collected by centrifugation (15000*g*, 10 min, 4 °C) and resuspended in 500 μL of trypsin digest buffer (1 mM magnesium acetate, 0.1 mM EDTA, 7.6% glycerol, 10 mM MOPS, pH 7.0, 0.1 mM dithiothreitol) supplemented with the appropriate pheromone ligand at 5 μM. TPCK-treated trypsin (Sigma Chemical, St. Louis, MO) was added to a final concentration of 30 μg/mL, and samples were incubated for various times at 30 °C. Parallel reactions were run on samples in the absence of trypsin to control for degradation that might occur during incubation. Digestion was terminated by addition of NaOH to a final concentration of 0.015 N. Membranes were collected by centrifugation in an Airfuge ultracentrifuge (Beckman Coulter, Fullerton, CA) and solubilized in SDS sample buffer. Proteins were resolved by SDS−PAGE (15% acrylamide) using prestained Kaleidoscope molecular weight markers (Bio-Rad Laboratories, Hercules, CA) transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA) and probed with affinity-purified antireceptor antiserum directed against the N-terminal domain of the α-factor receptor (*38*), which were kindly provided by James Konopka, State University of New York, Stony Brook, NY. The bands were visualized with the Western Lightning chemiluminescent detection system (Perkin-Elmer Life Sciences, Boston, MA).

RESULTS

Biological Activity and Binding of Ste2p Variants Produced by Mutageneis of Residues 262−270. Nine amino acids near the junction between TM6 and extracellular loop 3 (residues 262−270) were mutated by site-directed mutagenesis (Figure 1). All residues were mutated to alanine except residue 265, which is alanine in the native protein; in this case the residue was mutated to glycine. The biological activity of each mutant receptor was measured by the growth arrest assay, as described in the Experimental Procedures. The response of cells expressing each of the receptor constructs, except Y266A, increased linearly throughout the range of pheromone tested (Figure 2A). Excluding Y266A, the amount of α-factor required to produce a 15 mm zone of inhibition, a halo size in the middle of the dose response curve, was similar for all mutant receptors (Table 1, Figure 2A). Cells expressing the Y266A receptor did not respond to α-factor even using a large amount (8 μg) of pheromone (data not shown). The IC_{50} value for each mutant receptor was determined from competition binding assays (Figure 2B, Table 1). Ligand binding was reduced 2−4-fold compared to the WT by mutations F262A, A265G, and Y266A. Mutations of the remaining six residues resulted in an increased α-factor binding affinity (up to 5-fold), without any significant change in biological activity as determined by the growth arrest assays.

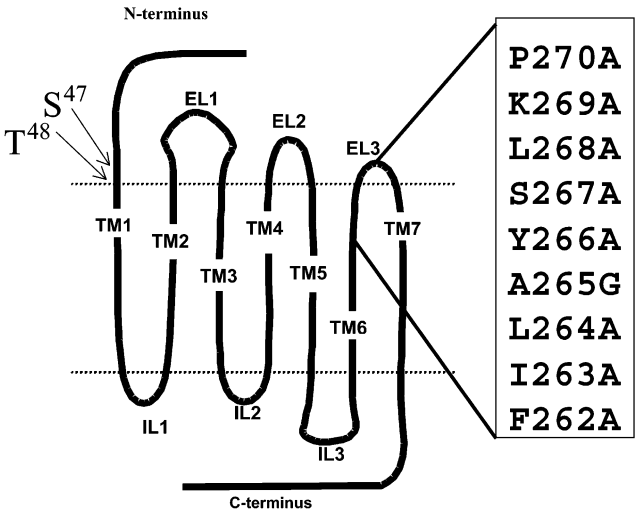

FIGURE 1: Topology of Ste2p and residues targeted for site-directed mutation. The predicted two-dimensional topology of the receptor is shown with the indicated extracellular domains (EL1, EL2, EL3), intracellular loops (IL1, IL2, and IL3), and transmembrane domains (TM1−TM7). Receptors containing the mutations indicated in the box were the subject of this study. Shown are two amino acid residues (S47 and T48) at the junction between TM1 and the N-terminus identified previously as contacts with the 10th residue (Q) of α-factor.

The Y266A Receptor Is Expressed at the Cell Surface. To determine whether the Y266A mutant receptor was expressed at the cell surface, whole cells (strain LM102) expressing the plasmid-encoded Y266A receptor or the WT receptor were incubated with a radiolabeled, photoactivatable ligand [Bpa^{1}Y^3(^{125}I)R^{7}F^{13} α-factor] and cross-linked by exposure to UV light as described previously (*16*). After the cross-linking, membrane proteins were resolved by SDS−PAGE. A radiolabeled band corresponding in size to the receptor−ligand complex (54 kDa) was detected, verifying the surface expression of the Y266A mutant (Figure 3).

Another experiment to measure the levels of cell surface expressed Y266A was done using a saturation binding assay on whole cells. This experiment demonstrated that the number of binding sites (B_{max}) was reduced in the Y266A receptor (∼14000 per cell) as compared to the WT Ste2p (∼40000 per cell) (Table 2). Previous studies indicate that 14000 binding sites per cell is sufficient for full signaling with Ste2p (*30*, *34*, *39*, *40*). This suggests that Y266A is expressed at levels fully capable of signal transduction if this receptor were indeed signaling-competent.

The Aromatic Ring Structure at Residue 266 Is Important for Receptor Function. To study the role of tyrosine at position 266 more thoroughly, five additional amino acid substitutions (Ser, Phe, Trp, Lys, and Leu) were made at this position and characterized by growth arrest and saturation binding assays to directly determine the ligand affinity (K_D) and number of binding sites (B_{max}) (Table 2, Figure 4). Substitution to lysine was not tolerated at this position and resulted in a complete lack of binding and biological activity. Although the Y266K receptor was verified by immunoblot analysis to be expressed at levels greater than the WT, the electrophoretic mobility of this mutant suggests that this receptor forms large amounts of aggregate material, and is most likely not correctly targeted to the cell membrane (data not shown). Substitution to serine or leucine produced

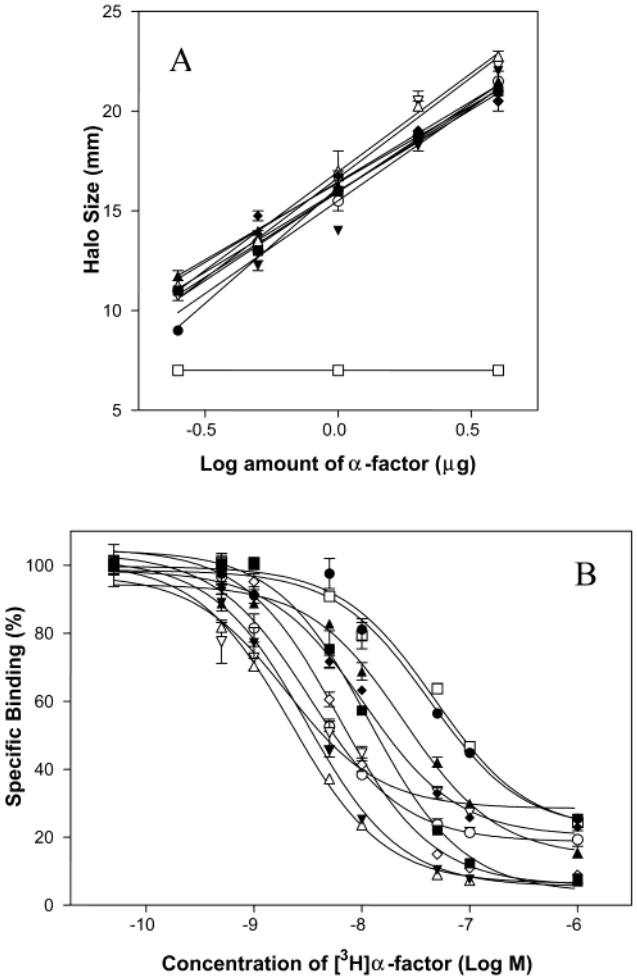

FIGURE 2: Biological activity and competition binding of α-factor in the Ste2p wild type and mutants in TM6. (A) The halo (zone of growth inhibition) sizes were measured at various concentrations of α-factor, and the data were plotted by regression analysis. The results are the mean SE of two separate experiments (see Table 1 for comparative activities). Key: WT (■), F262A (▲), I263A (▼), L264A (◆), A265G (●), Y266A (□), S267A (△), L268A (▽), K269A (◇), and P270A (○). (B) Yeast cells expressing the wild type or one of the mutant constructs were incubated with 6 nM [^{3}H]α-factor in the presence of increasing concentration of unlabeled α-factor as indicated on the abscissa. The experiments were repeated three times, and the results shown are the average SE of a representative experiment (see Table 1 for IC_{50} values). Key: WT (■), F262A (▲), I263A (▼), L264A (◆), A265G (●), Y266A (□), S267A (△), L268A (▽), K269A (◇), and P270A (○).

receptors with properties most similar to those of Y266A. Binding affinity was decreased 3−5-fold (K_D = 57.6 and 34.4 nM for Y266S and Y266L, respectively) compared to the WT, and the number of receptors expressed per cell was similar to (16500 for Y266S) or 3-fold greater than (37500 for Y266L) that of the Y266A mutant (14150). In contrast, substitution to aromatic residues resulted in near wild-type binding affinities (K_D = 10.5 and 16.5 nM for Y266F and Y266W, respectively). Although there was a decrease in receptor cell surface expression (B_{max} = 40000 binding sites/cell for the WT vs 25700 and 27700 binding sites/cell for Y266F and Y266W, respectively), these mutants retained full biological activity as assayed by growth arrest assay (88% and 92% of WT activity).

Mutation Y266A Confers a Strong Dominant-Negative Phenotype. A "dominant-negative" mutant masks the phe-

Table 1: Affinities and Biological Activities of Wild-Type and Mutant Receptors[a]

receptor	affinity of α-factor for receptor (IC_{50}, nM)	biological activity of receptor (% of WT)	receptor	affinity of α-factor for receptor (IC_{50}, nM)	biological activity of receptor (% of WT)
WT	11.8 ± 2.1	100 ± 11[b]	Y266A	47 ± 3.9	<5
F262A	24.3 ± 3.4	120 ± 11	S267A	2.3 ± 0.7	121 ± 15
I263A	3.2 ± 0.9	89 ± 6	L268A	2.1 ± 1.1	112 ± 9
L264A	10.5 ± 2.2	115 ± 13	K269A	6.2 ± 1.4	103 ± 7
A265G	38 ± 4.1	97 ± 8	P270A	3.4 ± 1.6	99 ± 8

[a] Affinities of various receptors for α-factor were measured by competition binding with 3[H]α-factor and expressed as IC_{50} values. Biological activities were calculated from the dose−response curves of a growth arrest assay. Results are expressed as mean ± SE of two to three separate experiments. [b] The amount of α-factor to produce a 15 mm halo was 0.68 μg for WT receptor.

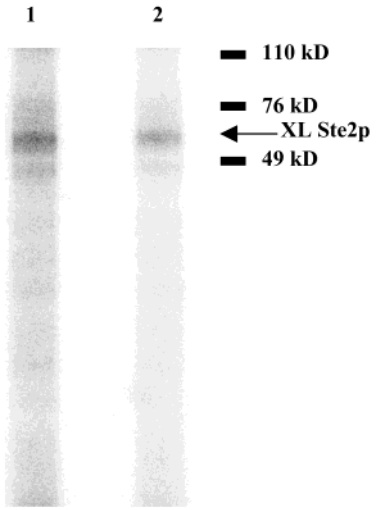

FIGURE 3: Photoaffinity labeling of Ste2p. Membranes derived from cells expressing wild-type (lane 1) or Y266A (lane 2) receptor were incubated with $Bpa^1Y^3(^{125}I)R^7F^{13}$ α-factor in the presence of UV irradiation. Membrane proteins were solubilized and run on SDS−PAGE. Labeled bands were detected by autoradiography.

Table 2: Affinities and Biological Activities of Ste2p with Substitutions at Y266[a]

	binding		
receptor	K_D (nM)	B_{max} (no. of binding sites/cell)	biological activity (% of WT)
WT	5.8 ± 1.4	40210 ± 3250	100 ± 9[b]
Y266A	40.5 ± 5.7	14150 ± 1070	<5
Y266S	57.6 ± 4.5	16500 ± 1100	<5
Y266F	10.5 ± 2.8	25700 ± 1540	88 ± 7
Y266W	16.5 ± 3.2	27700 ± 1800	92 ± 6
Y266K	ND[c]	NA[d]	<5
Y266L	34.4 ± 2.7	37600 ± 3020	<5

[a] Saturation binding assays were performed using whole cells expressing WT or mutant receptors. K_D and B_{max} values, calculated using a one-site binding model, are presented as the mean ± SE of at least three experiments. Each determination was performed in triplicate. [b] The amount of α-factor to produce a 15 mm halo was 0.65 μg for WT receptor. [c] ND = not detectable. [d] NA = not applicable. The number of binding sites per cell could not be determined. Cells expressing this receptor had undetectable levels of binding.

notype of the corresponding wild-type protein when both are coexpressed. We tested whether Y266A conferred a dominant-negative phenotype when coexpressed with WT

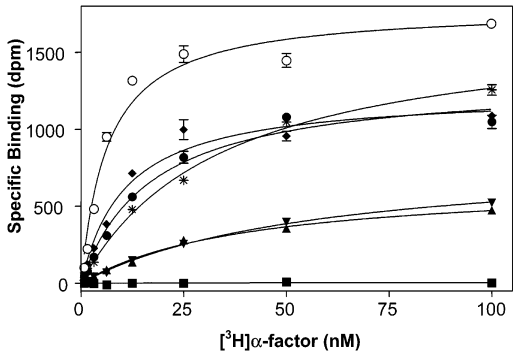

FIGURE 4: Saturation binding isotherms of [3H]α-factor for wild-type and various mutant receptors at position Y266. Cells harboring wild-type (○) and Y266F (◆), Y266W (●), Y266L (∗), Y266S (▼), Y266A (▲), and Y266K (■) mutant receptors were incubated with increasing concentrations of [3H]α-factor and assayed as described in the Experimental Procedures. Specific binding was determined by subtracting counts in the presence of excess cold α-factor. Assays were carried out at least three times, and each concentration point was performed in triplicate. The results shown are from a representative experiment with calculated K_D and B_{max} values presented in Table 2.

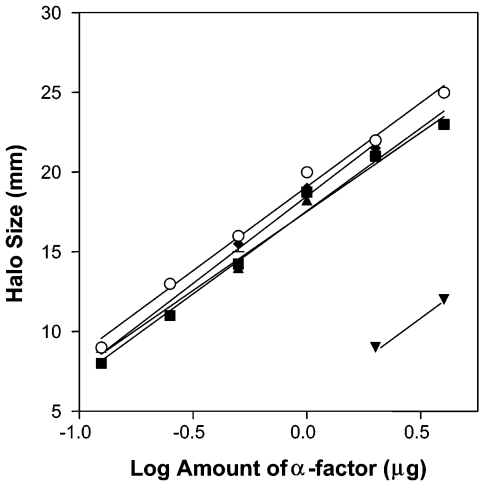

FIGURE 5: Dominant-negative effects of the Y266A mutant receptor on growth arrest. The indicated mutant or wild-type receptors were expressed in strain LM23-3az that contains a chromosomal copy of *STE2*. The growth arrest assays were conducted as described in the Experimental Procedures. The results are the average ± SE of two independent experiments. Key: LM23-3az expressing *STE2* from the chromosome (○, *cSTE2*), *cSTE2* coexpressed with plasmid-encoded *STE2* (■), F262A (▲), Y266A (▼), or K269A (◆).

receptor. Plasmids encoding the Y266A mutant receptor were expressed in LM23-3AZ, a strain containing a chromosomal copy of WT Ste2p. This same strain was also transformed with a plasmid encoding either the WT Ste2p or the mutant F262A or K269A. Coexpression of WT or F262A or K269A mutant constructs did not significantly influence halo formation. In contrast, when used in a growth arrest assay (Figure 5), strains coexpressing the WT and the Y266A mutant were much less sensitive to the pheromone, compared to the WT control, requiring more than 10 times the amount of ligand to produce a detectable halo. This dominant-negative phenotype could be suppressed by coexpression of the Y266A mutant construct with pMD82, a plasmid which overexpressed the G protein subunits. In this case, sensitivity to the pheromone was restored, and dose-dependent halos were

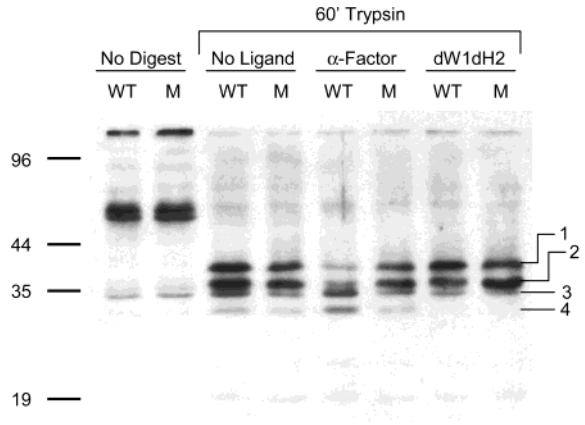

FIGURE 6: Partial tryptic digest of wild-type and Y266A mutant receptors. Immunoblot of membranes (20 μg/lane) incubated with either native α-factor or [desW^{1}desH^2]α-factor prior to trypsin digestion (60 min, 30 °C). Parallel controls were completed in the absence of ligand or trypsin. Molecular size markers (kDa) are indicated at the left. Four major digest products (1−4) are indicated on the right.

generated in growth arrest assays using low amounts (0.25−2 μg) of ligand (data not shown).

Y266 Is Essential for Conformational Change of the Receptor upon α-Factor Binding. Membranes expressing either the WT or Y266A mutant receptor were subjected to partial tryptic digest in the absence of ligand or in the presence of either α-factor or [desW^{1}desH^2]α-factor, an α-factor antagonist. The cleavage products were resolved by SDS−PAGE using 15% Tris−glycine gels, immunoblotted with an antibody directed against the N-terminal domain of the receptor and visualized by chemiluminescence. In the absence of trypsin, a doublet corresponding in size to Ste2p (approximately 50 kDa) is the major species present, with a less intense band at approximately 35 kDa (Figure 6). The smaller band is likely the result of degradation during the preparation of the membranes as observed in previous studies (*16*, *37*). Digestion with trypsin for 60 min in the absence of ligand resulted in the generation of four major fragments, labeled as 1−4. No difference in digestion pattern was observed when the WT was compared to the Y266A mutant receptor in the absence of ligand. Digestion in the presence of α-factor rendered the WT receptor more susceptible to digestion, in agreement with results previously reported (*37*). Under these conditions, bands 1 and 2 became less intense, while product accumulated in bands 3 and 4. In the presence of [desW^{1}desH^2]α-factor, the WT receptor was less readily digested; the banding pattern was similar to that observed for the unoccupied receptor; however, band 4 was absent. The results for the Y266A mutant receptor are somewhat different. As noted above, in the absence of ligand, the digestion pattern was very similar to that observed for the WT receptor, suggesting that both have similar topologies in the ligand-unbound, or inactive, state. In the presence of α-factor, the digestion pattern of the Y266A mutant was the same as that of the unoccupied mutant receptor. Upon binding the antagonist, the Y266A receptor digestion pattern changed and was similar to that observed for antagonist bound to the WT receptor.

To determine whether the differential sensitivity of the Y266A receptor was due to an intrinsic difference in receptor structure or was related to the nature of its interaction with the G protein, the tryptic digest was completed in the diploid background CG990. In diploid cells, the mating loci are silent; thus, these cells do not express G proteins. The WT and Y266A mutant constructs were expressed under the control of the constitutive GPD promoter, since the native *STE2* promoter would not be active in a diploid background. As was observed in the haploid background, in the presence of α-factor the tryptic digest pattern of the Y266A receptor did not change; bands 1 and 2 remained resistant to digestion (data not shown). This suggests that the differential trypsin sensitivity of the mutant when compared to the WT receptor was due to a change in receptor structure, rather than in the nature of its interaction with the G protein.

Table 3: Affinities and Biological Activities of Ala-Scanned α-Factor Analogues for Wild-Type and Y266A Mutant Receptors[a]

	binding (K_i, nM)		fold	biological activity[b] (%)	
peptide	WT	Y266A	change	WT	Y266A
α-factor	6.8 ± 1.5	41 ± 4.2	−6.0	100 ± 11	<5
[Ala^1]	216 ± 18	96 ± 10	+2.3	110 ± 12[c]	<5
[Ala^2]	505 ± 33	132 ± 12	+3.8	38 ± 4	<5
[Ala^3]	250 ± 16	63 ± 6	+4.0	13 ± 2	<5
[Ala^4]	365 ± 21	110 ± 17	+3.3	14 ± 2	<5
[Ala^5]	8 ± 2	58 ± 4.5	−7.2	124 ± 12	<5
[Ala^6]	1560 ± 110	3420 ± 175	−2.2	43 ± 5	<5
[Ala^7]	27 ± 2.5	280 ± 17	−10.4	141 ± 13	<5
[Ala^8]	9 ± 2	62 ± 5	−6.9	68 ± 6	<5
[Ala^9]	2150 ± 160	5590 ± 410	−2.6	13 ± 2	<5
[Ala^{10}]	570 ± 30	2840 ± 135	−5.0	45 ± 5	<5
[Ala^{11}]	140 ± 17	1110 ± 70	−8.0	70 ± 6	<5
[Ala^{12}]	1050 ± 90	11100 ± 1180	−10.6	53 ± 5	<5
[Ala^{13}]	4900 ± 450	16600 ± 1700	−3.4	23 ± 11	<5

[a] K_i values for α-factor and 13 Ala-scanned analogues were determined in competition binding assays by displacement of [^{3}H]α-factor. Biological activities were measured by the growth arrest assay. All values are the mean ± SE from three separate experiments. [b] The amount of α-factor to produce a 15 mm halo was 0.62 μg for WT receptor. [c] This halo was not clear and disappeared within 36 h.

Mutation of Y266A Alters Binding of N-Terminally Modified α-Factor Analogues. To further probe the uncoupling of ligand binding and signal transduction in the Y266A receptor, a wide variety of α-factor analogues, including antagonists and partial agonists, were tested for biological activity in an effort to determine whether any particular feature of the ligand could compensate for the receptor defect. Thirteen Ala-scanned analogues of α-factor were tested in binding and growth arrest assays (Table 3). These analogues had a wide spectrum of growth arrest activity, when tested using WT receptor, ranging from partial to enhanced agonist. In contrast, none of the 13 analogues were able to induce growth arrest in cells expressing the Y266A mutant receptor. In the competition binding assay, the Ala-scanned α-factor analogues exhibited a range of affinities, reported as K_i values, for binding to the Y266A receptor. Alanine substitution in the first four residues of the ligand ([Ala^1]- through [Ala^4]α-factor) resulted in pheromone that bound better to the Y266A receptor than to the WT. For example, for the [Ala^1]α-factor, in which the native tryptophan residue was replaced with alanine, the binding affinity (K_i) for the WT receptor was 216 nM, approximately 30-fold less than the binding affinity of native ligand to the WT receptor (6.8 nM). The binding affinity of this same ligand to the Y266A receptor was 96 nM, indicating an increase in binding affinity (216 nM for WT vs 96 nM for Y266A). Similar changes

Table 4: Binding Affinities of Antagonists and α-Factor Analogues[a]

peptide	WT	Y266A	fold change
α-factor	6.5 ± 2	42 ± 4	−6.5
[desW[1],desH[2]]α-factor	67 ± 7	19 ± 3.5	+3.5
[desW[1],A[3]]α-factor	84 ± 7	61 ± 5	+1.4
[Y[1]]α-factor	121 ± 13	30 ± 4	+4.0
[D-A[9]]α-factor	6.0 ± 1.8	48 ± 4.5	−8.0
[Orn[10]]α-factor	205 ± 19	940 ± 83	−4.0

[a] K_i values (nM) obtained from competition binding assay using [^{3}H]α-factor were the mean of two separate experiments, each of which was performed in triplicate.

(2−4-fold increases in affinity) were observed for the [Ala[2]]- through [Ala[4]]α-factor analogues. Although none of the [Ala[1]]- through [Ala[4]]α-factors had a better affinity than native ligand for the WT receptor, all of these N-terminally substituted ligands bound better to the mutant receptor than they did to the WT receptor. In contrast, [Ala[5]]- through [Ala[13]]α-factors exhibited 2−10-fold decreases in their affinities for the mutant receptor in comparison to their affinities for the WT Ste2p. These decreases were similar in magnitude to the decrease in affinity exhibited by α-factor for the Y266A mutant in comparison to WT Ste2p (6-fold).

To further examine the increased binding affinity which resulted upon changes in the N-terminus of the ligand, α-factor analogues modified by substitutions other than alanine, [Y[1]]-, [D-Ala[9]]- and [Orn[10]]α-factors as well as the antagonists [desW[1],desH[2]]α-factor and [desW[1]Ala3]α-factor were tested (Table 4). Similar to the observation made for the [Ala[5]]- through [Ala[13]]α-factors, the affinity of [D-Ala[9]]- and [Orn[10]]α-factors for the Y266A receptor was decreased (8- and 4-fold, respectively) when compared to their affinity for the WT receptor. In contrast, the N-terminal substitution of tyrosine for tryptophan in the [Y[1]]α-factor resulted in a ligand with 4-fold increased affinity for the mutant receptor, similar to what was observed for the [Ala[1]]- through [Ala[4]]α-factors. The affinities of the antagonists for the Y266A receptor were also enhanced. The [desW[1]Ala[3]]α-factor analogue exhibited a slight increase in affinity (1.4-fold change), while [desW[1],desH[2]]α-factor showed a 3.5-fold change. Interestingly, the affinity of [desW[1],desH[2]]α-factor was the greatest of all ligands tested (K_i = 19 nM vs 42 nM for native α-factor). Despite the fact that these various substituted analogues bound to the Y266A receptor, none of these analogues were able to induce growth arrest (data not shown).

DISCUSSION

Recent studies of Ste2p indicated that residues in the sixth transmembrane domain play important roles in signal transduction to the heterotrimeric G proteins as a consequence of α-factor binding. For example, mutation of P258L in the center of this domain resulted in constitutive activation of Ste2p (*18*), suggesting that P258 may be involved in the switch from the inactive to the active form of the receptor. Interestingly, mutation of Y266C in TM6 was found to result in an inactivatable form of the receptor (*25*) despite the fact that mutagenesis of adjacent residues to Cys had no effect on signal transduction (*41*). However, no comprehensive studies regarding ligand interactions with this receptor domain have been performed.

Our group has been exploring binding site interactions between Ste2p and α-factor using molecular biological (*13*, *15*), spectroscopic (*42*, *43*), and photo-cross-linking (*16*) approaches. Evidence was found that α-factor interacts with residues near the extracellular face of Ste2p. Furthermore, binding sites for most peptide hormones are proposed to involve extracellular loops and the outer ends of transmembrane domains (*44*). Therefore, we decided to thoroughly explore the unique phenotype manifested by Y266C. Our strategy was to mutate Y266 and all residues from F262 to P270 to Ala, to evaluate signaling and pheromone binding by the resulting mutant receptors, and to investigate their interaction with selected α-factor analogues.

Pheromone-induced growth arrest and binding affinity analyses indicated that the majority of Ala substitutions in this critical receptor domain did not interfere with receptor function, with the notable exception of Y266A. Mutations at certain residues resulted in even better α-factor binding and/or increased signaling ability (Table 1). Although Ala mutation at Y266 yielded a receptor inactive for signaling, α-factor still binds to this receptor with relatively high affinity (Tables 1 and 2). The apparent reduction in the cell surface expression of Y266A receptor in comparison to WT Ste2p as shown from saturation binding experiments is not likely the source of the observed signaling-deficient phenotype. Our data showed that there is no clear correlation between expression levels and receptor function. For example, the inactive Y266L receptor has near wild-type expression levels whereas the highly active Y266F and Y266W receptors are reduced in expression by approximately one-third (Table 2). Furthermore, there is also no strict correlation between the K_D values of the receptor and signaling proficiency since Y266L and A265G have virtually identical K_D values despite the fact that the former receptor is inactive whereas the latter receptor exhibited wild-type signaling (Tables 1 and 2). Finally, it was previously shown that, unlike mammalian GPCRs whose function is regulated by cell surface expression levels, yeast cells that express anywhere from as low as 5% to as much as 20-fold excess of the normal level of receptors can transduce signal at near normal levels (*19*, *45*). Many studies measuring levels of Ste2p expression by saturation binding experiments have reported that between 6000 and 10000 receptors are expressed on the cell (*30*, *34*, *39*, *40*), making the WT Ste2p expression levels seen in this report unusually high. If the Y266A receptor were functional, 14000 receptors/cell should be more than sufficient to initiate signal transduction.

When coexpressed with WT receptor, Y266A strongly interfered with the WT signaling activity (Figure 5); this is consistent with previous identification of the Y266C receptor as a dominant-negative mutant (*22*). On the basis of the work presented by Leavitt et al. (*23*), the expression level of chromosomally encoded WT receptor was unaffected even when the Y266C dominant-negative receptor was coexpressed 20-fold on a multicopy plasmid. Since Y266A was expressed using a low-copy-number (CEN) plasmid, under its native promoter, it is expected that the ratio between WT and Y266A would be very similar. Although receptor dimerization could be a possible explanation for the dominant-negative phenotype, the ability to sequester G proteins from wild-type receptor by dominant-negative mutations has been experimentally confirmed in the yeast α-factor receptor system (*22*−*24*). Our current finding that overexpression of the G-protein subunits reverses the Y266A dominant negative

phenotype lends additional credence to the postulate that G protein sequestration may be the mechanism underlying this phenotype. Additionally, the observation that a dominant-negative mutant in the α-adrenergic receptor, which specifically inhibited signaling via Gqa, was rescued by the overexpression of Gqa (*46*) further supports this postulate. On the basis of this scenario, we propose that the signaling deficiency of the Y266A receptor is due to its inability to activate G proteins. More specifically it seems likely that Y266A mutation prevents Ste2p from assuming the activated state of Ste2p induced by α-factor binding.

A previous study demonstrated that Ste2p assumed unique topologies in the ligand-free, agonist-bound and antagonist-bound states as judged by trypsin digest patterns found by SDS−PAGE. We, therefore, tested the Y266A mutant receptor using this approach. The relatively high affinity for α-factor binding indicated that, in the resting state, the mutant receptor had a conformation similar to that of WT Ste2p. Supporting this conclusion, the tryptic digest of unoccupied Y266A receptor was nearly identical to that of WT Ste2p (Figure 6). Upon binding α-factor, the WT receptor underwent a shift in conformation, resulting in an increased sensitivity to trypsin, as previously reported (*37*). Although the active site of Y266A was saturated with pheromone, the digestion pattern was the same as that of the ligand-free state. The digestion pattern was independent of the interaction of the mutant receptor with G proteins; essentially identical results were observed in diploid cells that do not express G proteins. This indicates that mutation of Y266 to Ala prevented Ste2p from undergoing the conformational change necessary for subsequent signal transduction in response to ligand binding. Additionally, binding of the desW^{1}desH^2 antagonist to both WT and Y266A mutant receptors resulted in a tryptic digest pattern distinct from either the resting or agonist-bound state. This suggests that antagonist binding resulted in a novel conformational state, one differing from those of both the unoccupied and the agonist-bound receptor. Similar results were also reported for binding of the antagonist [desW^{1}A^3]α-factor to WT receptor (*37*).

Additional mutations at Y266 revealed that signal transduction required an aromatic ring moiety at this position since substitution of this residue with other aromatic amino acids (Y266F and Y266W) maintained high-affinity binding and receptor signaling ability. A specific interaction between an aromatic side chain of residue 266 and residues from other TMs may be critical for the induction or stabilization of the activated conformation of the receptor since neither the aliphatic leucine nor cationic lysine substitution could support receptor function (Table 2). In fact, the Y266C mutant receptor was found, in an allele-specific manner, to restore function in the nonsignaling E143K mutation in the third transmembrane domain. This was interpreted as indicating a specific interaction between residues E143 and Y266 in the third and sixth transmembrane domains of Ste2p, respectively (*25*). Although the critical involvement of hydrophobic residues at TM6 in receptor function was reported for other GPCRs (*47*), the Y266A mutant receptor differs from those previously characterized in that it appears to affect primarily signaling by the receptor, while having little effect on agonist binding.

To gain more insight into the nature of the Y266A signaling defect, various α-factor analogues, including antagonists, were tested for their binding and growth arrest activity. The Y266A mutation markedly impaired the binding of α-factor analogues with single alanine substitutions in positions 5−13, while the same substitution at positions 1−4 of α-factor showed increased binding affinity for this mutant compared to WT Ste2p (Table 3). Moreover, the antagonist [desW^1,desH^2]α-factor had the highest affinity for this mutant receptor (2.5-fold better binding compared to that of native α-factor, Table 4). However, none of these analogues resulted in growth arrest of the yeast cell. It appears that in addition to its involvement in the Ste2p activation pathway Y266 likely contributes to receptor−ligand interactions. Specifically, our data would be consistent with a direct interaction between Y266 and residues near the N-terminus of α-factor. It is difficult to explain how a single substitution in the receptor can influence binding with four successive residues (residues 1−4) in the ligand. It is possible that, rather than interfering with a direct contact, changing Y266 to alanine results in a significant but localized perturbation of this region of Ste2p at the membrane interface. Others have shown that aromatic residues such as Trp and Tyr influence the insertion of transmembrane helices into bilayers (*48*) and may play a key role in keeping the transmembrane domains of integral membrane proteins in proper register with respect to the membrane. Alternatively, the recognition of the N-terminal end of α-factor by Ste2p may be mediated by a conformational compatibility mechanism, as shown in the neurokinin receptor system (*49*). In this view, the outcome of mutations in the receptor or substitutions in the ligand would be reflected in a regional conformational change in the receptor binding site or ligand, rather than disruption of specific contacts between receptor and ligand. Y266 would be a key residue involved in the recognition of aspects of the conformation of the N-terminus of α-factor, thus allowing the agonist to bind productively and trigger a transition into an activated state.

The important role of an aromatic cluster in receptor activation and ligand binding has been described for peptide hormone receptors (*50*−*52*) as well as for the dopamine receptor (*53*). Since the N-terminus of α-factor is highly aromatic and hydrophobic (W^{1}HWL^4), this domain probably binds to hydrophobic residues comprising the receptor pocket. In addition to Y266 two aromatic residues (Y203 and F204) at the EL2−TM5 junction would be reasonable candidates for interaction with the N-terminus of α-factor. Interestingly, mutation of these residues also resulted in dominant-negative mutants (*22*).

The Y266A mutant receptor maintained relatively high agonist binding, but was deficient with respect to signaling activity. Interestingly, this same mutant receptor exhibited enhanced antagonist affinity, and to the best of our knowledge, this is the first report of a mutation in a G protein-coupled receptor to possess all of these characteristics. It is striking that the [desW^{1}desH^2]α-factor antagonist binds more strongly than the WT agonist to the Y266A receptor (Table 4). Considering the fact that WT Ste2p binds α-factor 10-fold more avidly than it does the desW^{1}desH^2 antagonist, the Y266A mutant appears to prefer this N-truncated antagonist. Furthermore, the Y266A receptor displays increased affinity for other N-terminally modified ligands, including [desW^{1}A^3]α-factor, [Y^1]α-factor, and the position 1−4 Ala-scanned α-factor analogues. This suggests that the

structure of the Y266A mutant receptor better accommodates analogues in which the overall hydrophobicity at the N-terminus has been reduced by either substitution or elimination of hydrophobic residues. Interestingly, all antagonists of Ste2p discovered to date fall into this category of α-factor analogue; therefore, Y266A appears to possess a binding site that would be highly favorable to Ste2p antagonists. Currently GPCRs are a major drug target representing nearly 50% of current drugs under development (*54*, *55*). Often the pharmaceutical agent acts as an antagonist to prevent undesired effects from excessive receptor signaling. Thus, factors that result in antagonist selectivity may be relevant to drug design, and structural studies of this type of mutant receptor may be highly significant.

In conclusion, our results indicate that the Y266A mutation in TM6 of Ste2p abolished agonist-induced growth arrest, without compromising high binding affinity for α-factor. The loss of signaling is thought to result from the inability of the mutant receptor to achieve the active state required for productive interaction with its cognate G protein. We also report that, upon binding ligand, the conformation of Y266A receptor remained unchanged and did not resemble the tryptic digest pattern observed in agonist-bound WT receptor. Finally, we provide evidence that Y266 likely interacts with the amine terminal residues of α-factor (W^1HWL^4) possibly via interactions involving aromatic side chains. Biophysical and biochemical investigations focusing on the N-terminus of α-factor and its interactions with other site-directed mutants of the receptor are under way.

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