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The carboxy-terminal tail of the Ste2 receptor is involved in activation of the G protein in the *Saccharomyces cerevisiae* α-pheromone response pathway

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Abstract

The Ste2 gene encodes the yeast α-pheromone receptor that belongs to the superfamily of seven-transmembrane G protein-coupled receptors. Binding of pheromone induces activation of the heterotrimeric G protein triggering growth arrest in G1 phase and induction of genes required for mating. By random PCR-mediated mutagenesis we isolated mutant 8L4, which presents a substitution of an asparagine residue by serine at position 388 of the α-factor receptor. The 8L4 mutant strain shows phenotypic defects such as: reduction in growth arrest after pheromone treatment, diminished activation of the *Fus1* gene, and impaired mating competence. The asparagine residue lies in the second half of the intracellular protruding C-terminal tail of the receptor, and its replacement by serine affects interaction with both the Gα and Gβ subunits. Since expression of the receptor as well as its kinetic parameters, i.e., ligand affinity and receptor number, are unaffected in the mutant strain, we propose that association of the C-terminal tail of the receptor with Gα and Gβ subunits is required for proper activation of the heterotrimeric G protein. Besides its described role in downregulation and in formation of preactivation complex, the results here shown indicate that the C-terminal tail of the receptor plays an active role in transmitting the stimulus of mating pheromone to the heterotrimeric G protein.

Keywords: Ste2; C-terminus; G protein; Signal transduction; Structure–function; Yeast; *Saccharomyces cerevisiae*

1. Introduction

Mating pheromone induces a complex response in *Saccharomyces cerevisiae* haploid cells that leads to growth arrest, induction of transcription of several genes, including *Fus1*, changes in morphology, and conjugation with a similarly induced haploid cell of the opposite mating type. Mating pheromone binds to cell type-specific receptors, Ste2p in *Mat**a*** cells [1] and Ste3p in *Matα* cells [2] that are coupled to heterotrimeric G proteins. Upon binding of pheromone, receptors catalyze dissociation of G protein, giving Gα and Gβγ moieties. Gβγ in turn triggers cellular responses [3].

Activated receptors catalyze the exchange of GDP for GTP at the high-affinity binding site for guanine nucleotides, located in the α subunit, thus initiating a well-regulated dissociation–reassociation cycle. New light has been shed on the mechanisms of G protein activation by crystal structures of $G\alpha i_1\beta_1\gamma_2$ [4], $G\alpha_t\beta_1\gamma_1$ [5], and free $G\beta_1\gamma_1$ [6]. These data along with biochemical studies indicate that contact of ligand to the binding site of receptor induces conformational changes in transmembrane domains that are different in the activated or inactivated states of receptors [7]. Initial studies with the β-adrenergic receptors demonstrated that removal of residues from either end of the third intracellular domain uncoupled the receptor from Gs, and mutations within the second intracellular domain and at the proximal end of the C-terminal tail reduced the efficiency of coupling. In addition there are examples of C-terminally truncated peptide hormone re-

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ceptors that retain binding of cognate ligand with high affinity but are incapable of signal transduction by themselves [8]. Subsequently, point mutations and chimeric substitutions within these same regions continued to point to their involvement in direct interaction with the G protein. So far, this has been demonstrated for the biogenic amine receptors (adrenergic, muscarinic, serotonergic), peptide receptors (angiotensin), and rhodopsin [9].

Carboxy-terminal tails of serpentine receptors vary considerably in their amino acid sequence. In the case of the yeast α-pheromone receptor, the cytoplasmic carboxy-terminal tail extends at least 130 amino acid residues out from the membrane. Although the C-terminus of the receptor interacts physically with its associated heterotrimeric G protein [10,11], mutagenesis studies have shown that it is not required for ligand binding, but is involved in desensitization after pheromone treatment [12,13], and in formation of a preactivation complex with its associated G protein [11].

Even though receptors lacking the full C-terminal tail are capable of signaling upon binding of α-pheromone, here we describe a point mutation in the C-tail of the α-factor receptor that diminishes coupling with the G protein by altering contact to both Gα and Gβ subunits.

2. Materials and methods

2.1. Strains and media

The yeast strains used in this study were: W303-1A (*Mat**a***, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, can1-100), W303-3A (*Mat**a***, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, can1-100, *ste2*::*Leu2*), W303-28 (*Mat**a***, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *sst1*, can1-100, *ste2*::*Leu2*), and 70 (*Mat**α***, *thr3*, *met1*). *Escherichia coli* strain DHα5 was used to propagate shuttle vectors and recombinant plasmids. YPD medium consisted of 1% yeast extract, 2% bacto-peptone and 2% glucose. SD minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SGal medium contained 0.67% yeast nitrogen base and 2% galactose. SD and SGal minimal media were supplemented with the required amino acids (50 μg ml^{-1}) to select for plasmid. 100 μM Cu^{2+} was used to induce constructs in the pCUP vector.

2.2. PCR-mediated random mutagenesis and mutant selection

The *Ste2* open reading frame (ORF) was subjected to random mutagenesis by PCR amplification as described [14]. 2 mM Mn^{2+} (instead of Mg^{2+}) and 250 μM dNTPs (except for dATP which was 125 μM) were used in the reaction. The *Ste2* ORF was amplified using oligodeoxynucleotides directed against the 5′ and 3′ ends that introduced at the same time *Nco*I (position 2) and *Asp*718 (15 nucleotides beyond the stop codon) restriction sites respectively. The PCR conditions were as follow: 5 min at 94°C, 50 cycles of 45 s at 94°C, 45 s at 55°C, and 60 s at 72°C, with a final extension of 10 min at 72°C. PCR products were digested with *Nco*I and *Asp*718 and subcloned into the pCUP vector previously digested with the same enzymes. This placed the Ste2 PCR products under the control of the CUP1 promoter. The *Ste2* mutants were selected as follows: W303-3A (Ste2p-less strain) was transfected with the pCUP-based library, plated on SD medium with amino acids and incubated for 48 h. Patches were replica-plated to YPD containing a lawn of *MAT*α cells and incubated at 30°C for 5 h. Mating patches were replica-plated to minimal medium and incubated at 30°C for 48 h to select for diploids.

2.3. Pheromone response assays

Response to α-factor was tested by monitoring cell number and induction of Fus1–LacZ fusion protein [14]. Strains to be tested were grown until the mid-exponential phase in selective medium. 5×10^7 cells were then transferred to selective medium containing 100 μM $CuSO_4$, to induce pCUP constructs. Finally α-pheromone was added at a final concentration of 1 μg ml^{-1}. Cell number was determined after 12 h incubation at 30°C using a cell counting chamber. The effect of pheromone over the mating pathway was determined by quantification of β-galactosidase activity as described [14].

2.4. Quantitative mating assays

Quantitative mating assays were done as follows. *Mat**a*** W303-1A and W303-3A containing the desired plasmids were grown until the mid-exponential phase in selective medium containing 100 μM Cu_2SO_4. 1×10^6 cells were mixed with 1×10^6 cells of the tester strain, collected on a nitrocellulose membrane filter, and placed on a YPD plate plus 100 μM Cu_2SO_4. Plates were incubated for 5 h at 30°C. The cells from each filter were suspended in water and plated on SD medium for diploid selection.

2.5. Construction of the GFP–Ste2 fusion proteins

A 0.7-kb PCR fragment of the gene encoding the plant version of the green fluorescent protein (GFP) was generated with oligodeoxynucleotides containing *Nco*I sites (at positions 2 and 415, respectively). This fragment was subcloned into pCUP–Ste2 constructs opened at *Nco*I. This gave an in-frame construction of GFP fused to the N-terminus of the Ste2 receptor under the control of the copper promoter.

2.6. FACScan analysis

GFP–Ste2 fusion receptors were detected by monitoring

fluorescence with a Becton Dickinson FACScan, using the CELL-Quest software. Transfected cells were grown overnight at 30°C in SD medium supplemented with the required amino acids and 100 μM Cu_2SO_4. Cells were collected, washed twice with cold water, and kept on ice while assayed.

2.7. Interaction assays

Assays of physical interaction were done with the LexA–B42 two-hybrid system [14]. The fragments encoding the C-terminal tail of wild-type and mutant *Ste2* were obtained by PCR introducing a restriction site for *Eco*RI at position 913. The PCR products were first cloned into pCR™II and then subcloned into pEG202 digested with the same enzyme. The full-length ORFs of *Gpa1* and *Ste4* were subcloned into pJG4-5 as in-frame *Eco*RI–*Xho*I fragments obtained by PCR amplification. The *S. cerevisiae* endochitinase gene (*Cts1-2*), amplified by PCR using oligodeoxynucleotides that introduce *Eco*RI and *Xho*I restriction sites, was subcloned into pEG202 for use as the interaction-negative control. Strain W303-1A was transfected with two-hybrid plasmids and grown in SGal medium at 30°C for 15 h. Protein interactions were determined by the ability of recombinant proteins to induce expression of the *LacZ* reporter gene, located in the pSH18-34 plasmid.

2.8. Binding assays of α-pheromone

^{35}S-Labeled α-factor was prepared and purified as previously described [15]. Pheromone binding assays were done as described [16]. [^{35}S]H_2SO_4 was from ICN Radiochemicals. Cells growing in SD medium were induced with 100 μM Cu_2SO_4 4 h before binding assays.

3. Results

The Ste2 receptor-encoding gene was mutagenized by PCR amplification using conditions that reduce fidelity of DNA synthesis by Taq DNA polymerase as described before [14]. In this set of experiments we determined an error frequency of 0.2%. Mutagenized Ste2 ORFs were subcloned into the Cu^{2+}-inducible vector pCUP, generating a library of about 200 independent clones. Based on the analysis of inserts from 10 randomly picked clones we estimated 80% recombinant plasmids in the pCUP library. The library was used to transfect the sterile strain *ste2*. In a first screening we assayed the transfected strain in a diploid formation assay, identifying defective Ste2p receptors by their inability to rescue the Ste2-lacking mutant from sterility. In this first approach, we found a broad spectrum of clones, from those that totally reversed the sterile phenotype of the *ste2* mutant to those that completely failed to restore mating. We focused on the clones that presented an intermediate phenotype from which we selected mutant 8L4 for further and detailed characterization. Sequence analysis of the 8L4 mutant showed a transition A to G at position 1163 that creates the replacement of N(388) by S. This residue is located in the putative C-terminal tail of the Ste2 receptor. The mating efficiency of the *ste2* mutant carrying the 8L4 allele of the receptor was estimated to be 45% of the strain carrying the wild-type receptor (Table 1). Expression of the mutant receptor did not affect mating of either the wild-type strain or the *ste2* strain expressing the wild-type allele of the receptor (not shown), which indicates the recessive character of the ste2$^{N(388)S}$ mutation.

Occupancy of receptor by pheromone induces activation of G protein via guanine nucleotide exchange in the Gα subunit. Free Gβγ dimer in turn activates a cascade of phosphorylations, which leads to growth arrest in G1 phase and triggers expression of genes required for diploid formation, one of which is *Fus1*. Here we used, as a reporter of the pheromone-activated pathway, a fusion protein of Fus1p with the bacterial β-galactosidase whose ex-

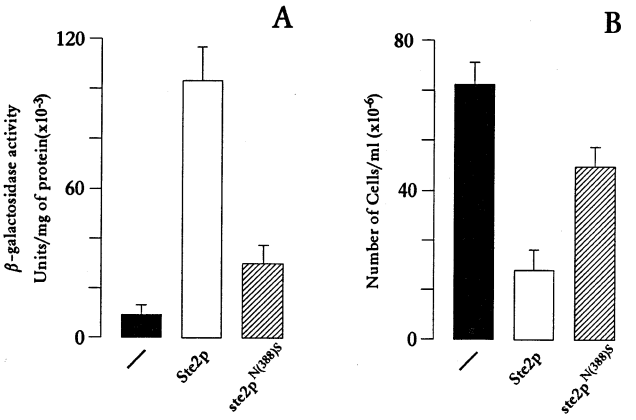

Fig. 1. Effect of α-pheromone on *Fus1* induction and growth properties of *ste2* cells carrying pCUP constructs. Cells harboring plasmids were grown until mid-exponential phase in SD plus the required amino acids. 5×10^7 cells were transferred to 2 ml of selective medium containing 100 μM Cu_2SO_4. α-Pheromone was added at a final concentration of 1 μg ml^{-1}. Cultures were incubated for 12 h at 30°C. A: β-Galactosidase activity. B: Cell number. Solid bar, no receptor. Open bar, wild-type receptor. Hatched bar, 8L4 receptor.

Table 1
Mating efficiency of strain *ste2* carrying pCUP constructs

Plasmid	Mating efficiency[a]
pCUPSte2	1.00
pCUPste2$^{N(388)S}$	0.45
pCUP	0.00

[a]Numbers are relative to mating efficiency of strain W303-3A (*Mat**a**, ade2, his3, leu2, trp1, ura3*, can1-100, *ste2*::*Leu2*) transfected with the wild-type *Ste2* gene under the control of the Cu^{2+} promoter. Cells of the strain to be mated were combined with cells of the tester strain (70), on nitrocellulose membrane filters placed on the surface of a YPD plate containing 100 μM Cu_2SO_4 and incubated at 30°C for 5 h. Cells were diluted and plated on SD medium. Mating efficiency is defined as the number of diploids divided by the number of haploids of the strain being tested.

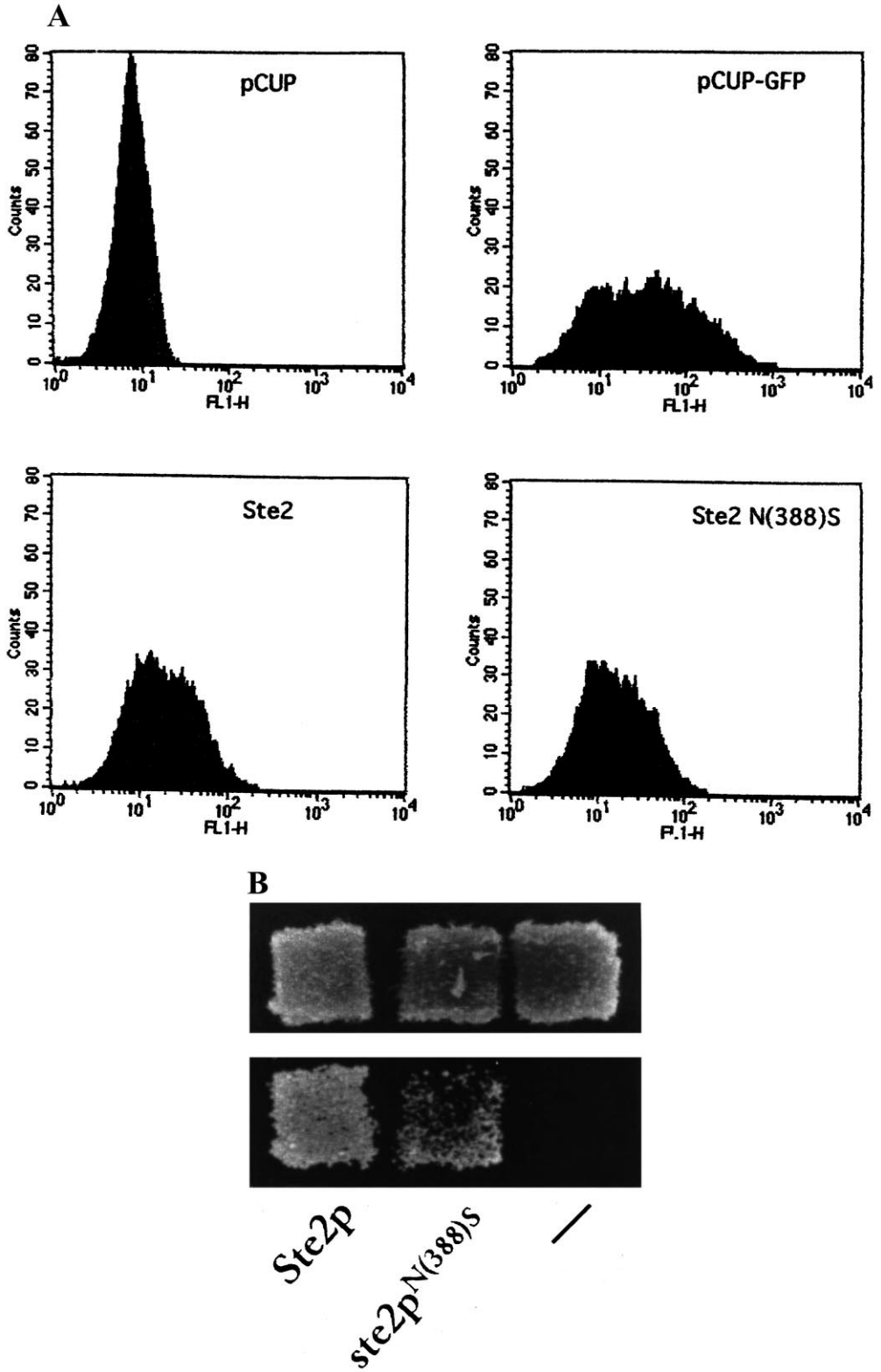

Fig. 2. A: Detection of GFP-fused wild-type and 8L4 receptors by FACScan analysis. 5×10^7 transfected cells were grown overnight at 30°C in liquid SD medium supplemented with the required amino acids plus 100 μM Cu_2SO_4. Cells were collected and washed twice with cold water. Green fluorescent protein was detected by FACScan (fixing wavelengths at 420/520 nm). B: Mating of ste2 strain transfected with the indicated GFP-fused proteins. Strains were grown overnight as patches in SD selective medium plus 100 μM Cu_2SO_4. Patches were replica-plated to YPD containing a lawn of strain 70 (*Matα*) and incubated for 5 h at 30°C. Patches were replica-plated to SD minimal medium and incubated for 48 h at 30°C to select for diploids.

pression is under the control of the *Fus1* promoter [14]. As can be observed in Fig. 1A, the β-galactosidase activity of the strain carrying the 8L4 receptor is reduced by about 50%, indicating that the mutant receptor is deficient to induce expression of the *Fus1* gene upon stimulation with α-pheromone. This observation is consistent with the effect that the 8L4 receptor has in the induction of growth arrest, i.e., cultures of the strain expressing the mutant receptor showed about two times more cells than those expressing the wild-type receptor (Fig. 1B).

The low sensitivity to α-pheromone of the strain expressing the 8L4 receptor could be due to either an alteration in its expression and/or inability to reach the plasma membrane. To address this question we devised the following protocol to detect the mutant Ste2 receptor. A fusion was made between the Ste2 receptor and GFP as indicated in Section 2. Fluorescence of GFP was measured and its distribution was analyzed with a Becton Dickinson FACScan employing the CELL-Quest software. As controls we analyzed the distribution of the wild-type Ste2p–GFP fusion protein and GFP alone expressed under the control of the Cu^{2+} promoter. As seen in Fig. 2A, cells transfected with the pCUP–GFP plasmid displayed a clear shift to the right compared to control cells transfected with pCUP alone. The average fluorescence increased eight-fold, from 8 to 69 arbitrary fluorescent units (AFU). A significant fraction of the cell population transfected with pCUP–GFP displayed fluorescence above control levels. 63% of the cell population transfected with pCUP–GFP displayed a fluorescence intensity higher than 25 AFU compared to only 0.5% of the cell population transfected with pCUP. In cells expressing the hybrid protein Ste2–GFP, the average fluorescence increased 3.1-fold (from 8 to 25 AFU), while in cells expressing the $Ste2^{N(388)S}$–GFP hybrid protein the average fluorescence increased 2.7-fold (from 8 to 22 AFU). In cells expressing either of the hybrid proteins a significant portion of the cell populations displayed a fluorescence intensity higher than 25 AFU, 45% for the Ste2–GFP hybrid and 39% for the Ste2*N(388)S*–GFP hybrid. From these results we draw two major conclusions: (a) hybrid proteins are overexpressed with respect to controls, but to lower levels than GFP alone (this effect could result from the normal regulation that controls the endogenous Ste2 protein level imposed over the hybrid proteins), and (b) both wild-type and mutant Ste2 hybrid proteins display similar expression levels. On the other hand, the intensity of fluorescence depended directly on the dose of Cu^{2+} (not shown).

In addition, wild-type receptor fused to GFP is able to fully reverse the mating deficiency of the *ste2* null mutant (Fig. 2B). This functional test indicates that the hybrid protein retains its ability to reach the plasma membrane and to activate the pheromone response pathway. In addition, the 8L4–GFP fusion protein is able to mediate mating at the same level as the 8L4 receptor alone does (Fig. 2B). The results shown above indicate that the N(388)S mutation does not alter cell surface expression of the 8L4 receptor.

Table 2
Agonist binding affinity and cell surface expression of wild-type and mutant receptors

Ste2 allele	K_d (nM)	B_{max} (sites per cell)
Wild-type	1.5 ± 0.5	4200 ± 400
$ste2^{N(388)S}$	1.9 ± 0.7	3600 ± 600
ste2Δ	n.d.	n.d.

The indicated Ste2 alleles were expressed from the pCUP plasmid in strain W303-28 (*Mat**a**, ade2, his3, leu2, trp1, ura3, sst1*, can1-100, *ste2*::*Leu2*). Cells were treated with radiolabeled α-pheromone for 2 h at 30°C. Values were calculated by nonlinear regression of data obtained from three independent transformants assayed by duplicated. n.d., specific binding not detected.

To determine whether the N(388)S mutation in the α-pheromone receptor affects ligand binding we carried out equilibrium binding studies with intact cells. The affinity for α-pheromone (K_a), measured as a factor of the apparent equilibrium dissociation constant ($1/K_d$), and the steady-state levels of cell surface receptors (B_{max}) are shown in Table 2. The 8L4 mutant receptor possessed affinities for α-factor (K_d = 1.2–2.6 nM) that were identical to the values obtained for the wild-type receptor (K_d = 1.0–2.0 nM). These values are essentially the same as those previously published [15,17]. The B_{max} values indicate that wild-type receptor varied between assays from 3800 to 4600 receptors per cell and 8L4 mutant receptor varied between 3000 and 4200 receptors per cell.

The results shown above indicate that the defective ability of the ste2p mutant receptor to mediate growth arrest and mating induction caused by the point mutation in its C-terminal tail could not be accounted for either by alteration in its kinetic constants or by protein destabilization. Therefore the deficient activity of the 8L4 receptor should be due to failure to activate adequately the heterotrimeric G protein.

Since N(388) is located in the C-terminal cytoplasmic tail of the Ste2 receptor, we measured the ability of mutant and wild-type C-terminal domains to bind Gα (Gpa1p) and Gβ (Ste4p) subunits employing the two-hybrid system. The fragment encoding the C-terminal tail of the Ste2 receptor was subcloned into pEG202 plasmid in frame with the *LexA* DNA binding domain, placing the hybrid protein under the control of the constitutive *Adh1* promoter. *Gpa1* and *Ste4* were subcloned into pJG4-5 plasmid fused to the B42 transcription activation domain, which is expressed with the *Gal1*-inducible promoter. Fig. 3 shows β-galactosidase activity induced by interacting pairs. The two-hybrid assay detected that the C-terminal tail of the 8L4 receptor has a reduced capacity (50%) to associate with both Ste4p and Gpa1p subunits. This is true for three independent clones of each interacting pair assayed in duplicate. Control experiments made with the mutant and wild-type Ste2-COOH fragments as binding

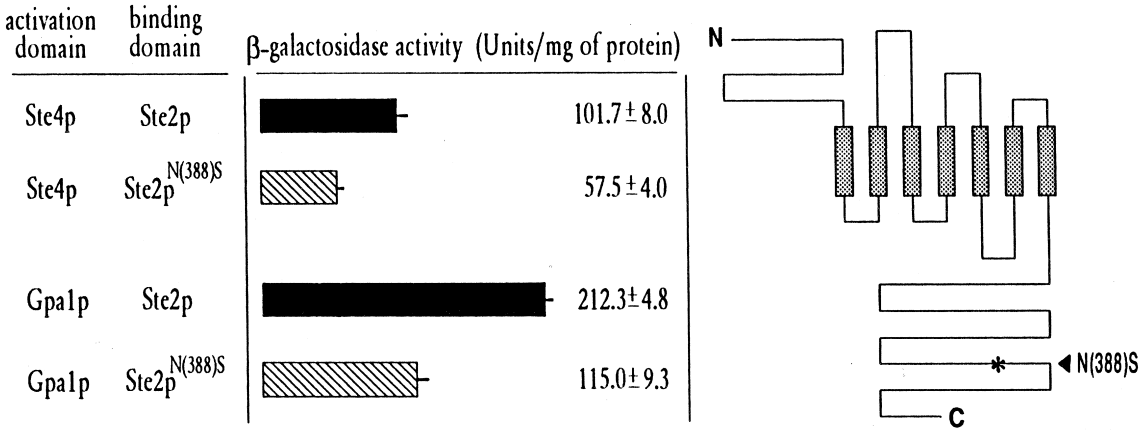

Fig. 3. Physical association of wild-type and 8L4 receptors to Ste4p and Gpa1p subunits. Strain W303-1A transfected with the plasmids indicated in Section 2 was grown until the mid-exponential phase in SD selective medium. 5×10^7 cells were transferred to liquid SGal medium and incubated at 30°C for 15 h. The β-galactosidase activity corresponds to the average value of three independent clones assayed in duplicate. The activation domain corresponds to B42-fused proteins cloned into pJG4-5. The binding domain corresponds to LexA-fused proteins cloned into pEG202. A diagram of the receptor indicating localization of the N(388)S mutation is shown.

domain measured alone and with the *S. cerevisiae* endochitinase (Cts1p) as activation domain showed β-galactosidase activities ranging from 0.5 to 4 U mg^{-1} of protein (not shown). These data clearly indicate that replacement of the N(388) residue results in reduced contact between the C-terminal tail of the receptor with both subunits of the G protein affecting the transmission of the pheromone stimulus.

4. Discussion

Several studies with a variety of G protein-coupled receptors have suggested that not only their intracellular loops but also their carboxy-terminal tails play a crucial role in maintaining a productive G protein coupling. Crystal structures of heterotrimeric G protein as well as biochemical and mutagenesis studies indicate that the C-terminal tail of the Gα subunit interacts directly with the receptor perhaps in a domain formed by transmembrane regions 3, 6, and 7 [10,18]. In this model the receptor's carboxy-terminal tail may be accommodated in the interface between Gα and Gβγ interacting with both subunits.

In this work we describe that the replacement of N(388) by S in the C-terminal tail leads to reduced contact between the α-pheromone receptor and the Gα and Gβ subunits, affecting activation of the G protein upon binding of pheromone. Substitution of N by S (i.e., changes in mass and van der Waals volume) at this particular position disrupts a putative β-sheet structure located at residues 383–393. This suggests that the N(388) residue, rather than being a contact point to both subunits of the G protein, is essential to maintain the binding structure of the C-terminal tail to both Gα and Gβ subunits. N(388) lies within residues 360–431, which have been implicated in the formation of receptor–G protein preactivation complexes [11]. Taking this into account, the recessive nature of the N(388)S mutation indicates that the C-terminal tail of the mutant receptor is defective in promoting the formation of preactivation complexes and suggests that these are a required step for G protein activation upon binding of pheromone.

The α-factor receptor undergoes a pheromone-dependent hyperphosphorylation of the C-terminal hydrophilic domain that mediates downregulation by pheromone-stimulated receptor endocytosis [19]. Although it was shown that the C-terminal domain of the α-factor receptor is unlikely to play an essential role in G protein activation, since truncated receptors lacking this region remain responsive to agonist [12,13], the results described here indicate that the C-terminal tail of the receptor, when present, plays an active role in signal transduction and may regulate G protein activation. In fact, removal of the C-terminus is not totally inert for receptor function, since one of the described truncations of the C-terminus (the so-called Δ408), which lacks the entire C-terminal tail, reduced mating to 50% [13].

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