

N-Terminal Residues of the Yeast Pheromone Receptor, Ste2p, Mediate Mating Events Independently of G1-Arrest Signaling

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ABSTRACT

In *Saccharomyces cerevisiae*, mechanisms modulating the mating steps following cell cycle arrest are not well characterized. However, the N-terminal domain of Ste2p, a G protein-coupled pheromone receptor, was recently proposed to mediate events at this level. Toward deciphering receptor mechanisms associated with this mating functionality, scanning mutagenesis of targeted regions of the N-terminal domain has been completed. Characterization of *ste2* yeast overexpressing Ste2p variants indicated that residues Ile 24 and Ile 29 as well as Pro 15 are critical in mediating mating efficiency. This activity was shown to be independent of Ste2p mediated G1 arrest signaling. Further analysis of Ile 24 and Ile 29 highlight the residues' solvent accessibility, as well as the importance of the hydrophobic nature of the sites, and in the case of Ile 24 the specific size and shape of the side chain. Mutation of these Ile's led to arrest of mating after cell contact, but before completion of cell wall degradation. We speculate that these extracellular residues mediate novel receptor interactions with ligand or proteins, leading to stimulation of alternate signaling effector pathways. *J. Cell. Biochem.* 107: 630–638, 2009. © 2009 Crown in the right of Canada

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Among the most thoroughly characterized signaling systems are those related to G protein-coupled receptors (GPCRs) in the plasma membrane. GPCRs are helical seven-transmembrane integral membrane proteins, which comprise one of the largest super-families of receptors in the human genome (~800) [Takeda et al., 2002] and represent a major class of pharmaceutical targets [Drews, 2000]. The classical function of GPCRs is to sense and translate stimuli into highly specific intracellular responses through conformationally mediated interactions with heterotrimeric G proteins [Pierce et al., 2002].

STE2 (α -factor mating receptor) and *STE3* (a-factor mating receptor) encode GPCRs presented in the plasma membranes of

haploid yeast cells [Bardwell, 2005; Elion et al., 2005; Slessareva and Dohlman, 2006]. *STE2* is expressed by *MAT α* cells and *STE3* by *MAT α* cells. Like all other GPCRs, activation of these receptors by agonist leads to well characterized G protein-mediated mitogen activated protein kinase signal transduction events. In yeast, these kinases activate G1 cell cycle arrest and polarized growth of a mating projection. Subsequent mating events are then initiated which include opposite mating type recognition and contact, cell wall remodeling, fusion and degradation, membrane fusion and nuclear fusion leading to vegetative zygotic growth. While mechanisms mediating the initial G1 cell cycle arrest stage are well characterized [Bardwell, 2005; Elion et al., 2005; Slessareva and

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Dohlman, 2006], mechanisms modulating the mating steps following G1 arrest, generally remain enigmatic.

Mutational screening of the extracellular domain of Ste2p has mainly focused on regions immediately adjacent to the ends of the transmembrane segments. These studies have identified a variety of residues that mediate receptor signaling leading to G1 cell cycle arrest [Lin et al., 2003, 2004, 2005; Hauser et al., 2007]. Of these, a number of aromatic residues, such as Phe 204 and Tyr 266, are specifically proposed to form a large number of contacts with α -factor modulating receptor activation and signaling events. Mutational analyses of N-terminal residues of Ste2p have been limited to sites proximal to TM1 extending out to residue 44 [Lin et al., 2004]. In this latter study, mutation of residues Val 45, Asn 46, Thr 50 and Ala 52 were found to lead to defective G1 arrest. In addition, mutational analyses of the N-terminal glycosylation sites, Asn 25 and Asn 32, indicated that these are not critical for signaling or mating [Mentesana and Konopka, 2001]. In contrast, a role in modulation of mating was recently proposed for the N-terminal tail of Ste2p [Shi et al., 2007]. Specifically, deletion of the first 20 amino acids (Δ N20) of Ste2p was found to decrease mating efficiency, but had little impact on receptor mediated G1 arrest. The mechanism by which the Ste2p N-terminal domain modulates this mating functionality remains to be determined.

Toward deciphering the mechanism by which the Ste2p receptor modulates mating efficiency, a systematic dissection of the site specific functional contributions of its N-terminal residues has been carried out. Targeted scanning mutagenesis methods, combined with cysteine accessibility studies and *in vivo* analyses have led to the identification of two highly conserved hydrophobic residues and a conformationally constrained Pro, all involved in modulating mating. The mating activity associated with these three sites was shown to be independent of receptor mediated G1 arrest signaling. Electron microscopy confirmed that mutation of these sites caused arrest of mating at a point after cell contact is made, but before cell wall degradation is completed. Potential mechanisms of mediation are discussed highlighting possible signaling strategies.

MATERIALS AND METHODS

CHEMICALS

Horseshoe peroxidase (HRP) conjugated anti-HisG antibody, anti-V5 antibody, FM4-64, Taq polymerase and *Bam*HI restriction endonuclease were all obtained from Invitrogen (Burlington, ON). *N*-dodecyl- β -D-maltoside (DM) was from Anatrace. α -factor was from Zymo Research. Turbo Pfu was obtained from Stratagene. All other reagents were from Sigma.

STRAINS AND PLASMIDS

E. coli strain DH5 α was used to propagate and amplify plasmids. *Saccharomyces cerevisiae* *MAT* α *his3-11, 15 leu2-3, 112 trp1-1 ura3-1 kan1-100 ade2-1 are1::HIS3* was a gift from S. Sturley (Columbia University) and *MAT* α *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 Δ STE2 (*MAT* α *ste2 Δ*) was obtained from the American Type Culture Collection. All PCR amplifications were carried out using Taq polymerase. Wild-type *STE2* was amplified from pTT/Ste2p-*

GFP [Shi et al., 2005] with forward primer 1 (5'-CGGCGGGGTA-CCGGATCCGCCACCATGTCTGATGCG-3') and reverse primer 2 (5'-CCGCCGGAATTCAGGATCCTCTAAATTATTATTATCTTCAGTCCAGAAC-3') and cloned into pYES2.1 (Invitrogen), which has a *GAL1* promoter, a *URA3* selectable marker and a 2 μ origin of replication to produce pYES2.1/Ste2p for expression in receptor null yeast. *STE2* N-terminal single site mutants were constructed using the Quick Change Site-Directed Mutagenesis kit (Stratagene), using pYES2.1/Ste2p as a template. The primers used for mutagenesis are listed in Supplemental Table I. For overexpression of Ste2p Cysless, Cysless-I24C and Cysless-I29C mutants in human embryonic kidney 293 EBNA-1 cells, DNA was amplified by forward primer 1 and reverse primer 2, using pYES2.1/Ste2p mutagenized plasmid as a template. The PCR fragments were digested by *Bam*HI and cloned into the pTT vector. All constructs were sequenced prior to transformation confirming the presence of target mutations and lack of any unexpected mutations.

HALO ASSAYS

The pheromone responses of pYES2.1/STE2 transformed yeast strains (*MAT* α *ste2 Δ*) were analyzed by halo assay. The strains were cultured overnight in SC minimal medium (-Ura) with 2% galactose and 1% raffinose. A 5 μ L sample of each resulting culture was mixed with 0.5% agar at 55°C and plated on the surface of a YPD plate with galactose. After solidification of the agar, the pheromone was added (2, 1, or 0.5 μ L of a 10 mM stock). The plates were inspected after 2–3 days incubation at 30°C.

FUS1 PROMOTER ACTIVITY ASSAY

STE2 mutants were quantitatively assayed for the ability to induce *FUS1-lacZ* expression using the fluorescent assay previously developed in the laboratory of F. Kippert [1995]. STE2 mutant expressing cultures were transformed with vector pSB234 for expression of *P_{FUS1}-FUS1(1–254)-lacZ* [Jin et al., 2008]. Essentially, 100 μ L of STE2 mutant expressing cultures were treated with 1 μ M α -factor, followed by a second dose of 500 nM α -factor 45 min later to replenish the degraded peptide. Negative controls for non-specific background signals were produced similarly, but without the addition of any pheromone. All samples were incubated at 30°C for 2 h, transferred to a 1.5 ml centrifuge tube, frozen in liquid nitrogen and then stored at –20°C. Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM Mg₂SO₄, 50 mM β -mercaptoethanol) containing 0.2% sarcosyl was added to the frozen samples to give a final volume of 600 μ L. The tubes were preincubated at 30°C for 30 min. One hundred fifty microliters of *O*-nitrophenyl- β -D-galactopyranoside (4 mg/ml) was added and tubes were briefly shaken by hand and further incubated at 30°C. After 2–3 h, the reactions were stopped by addition of 400 μ L of 1.5 M Na₂CO₃. The samples were centrifuged for 30 s at 16,000g and the absorbance of the supernatant was read at 420 nm. All assays were repeated at least three times. Arbitrary units were used to highlight relative values.

QUALITATIVE MATING EFFICIENCY ASSAY

WT *MAT* α SCY060 (Ura⁻, His⁺) was mated with *MAT* α *ste2 Δ* YFL026W, transformed with pYES2.1/Ste2p (encoding WT or

mutagenized STE2) vector (producing Ura⁺, His⁻ cells) and plated on media lacking uracil and histidine to select for mated diploid cells as follows. All strains were cultured overnight in 2% glucose and then, in the case of transformed MATa strains, in 2% galactose SC medium in the absence of uracil for 3 h. Cells were harvested by centrifugation at 2,500 rpm for 5 min, washed with sterile TE buffer (100 mM Tris buffer pH 7.5 with 10 mM EDTA), and resuspended in SC medium lacking histidine in the case of transformed MATa, and lacking uracil in the case of MAT α cells. To initiate mating, 5 \times 10⁶ cells of each mating type were collected and resuspended in 100 μ L of galactose media lacking histidine and uracil. Cells (5 \times 10⁴) as well as three- and ninefold dilutions were spotted on plates lacking histidine and uracil and incubated for 2–4 days at 30°C.

QUANTITATIVE MATING EFFICIENCY ASSAY

Strains of MATa expressing mutant and wild-type STE2 were grown individually overnight in glucose medium lacking uracil. Strains were harvested, re-suspended in galactose (2%) medium lacking uracil to OD_{600 nm} 0.4 and incubated at 30°C for 5 h. For each strain, 2 \times 10⁷ cells were mixed with 8 \times 10⁷ MAT α cells, filtered onto a 0.45 μ m membrane and washed with sterile water. Membranes were placed onto 2% galactose plates, incubated at 30°C for 5 h, and then approximately 200 MATa cells were plated on 2% glucose plates lacking both uracil and histidine for selection of zygote products and on SC plates lacking only uracil to quantify the MATa cells. Single colonies examined after 48 h incubation at 30°C and the ratio of plated colonies calculated as the quantitative mating ability of strains [Sprague, 1991]. The ratio of prezygotes to zygotes was assessed by observation of nuclear fusion in 4'-6-diamidino-2-phenylindole (DAPI) labeled cells, using a LEICA DMR fluorescence microscope, 40 \times objective, and filters with excitation/emission maxima of 360 nm/400 nm, and then compared to values for wild-type MATa cells.

ELECTRON MICROSCOPY

Samples for electron microscopy (EM) were prepared as described previously [Heiman and Walter, 2000], with minor modifications as follows. Equal volumes of fixative (1% glutaraldehyde, 0.2% paraformaldehyde, in 0.04 M pH 7.2 potassium phosphate buffer) and yeast suspension were mixed; fixation was for 4 h at 4°C. Fixed cells were suspended in warm 1% agarose, cooled, and washed in 0.9% (w/v) NaCl. Specimens were post-fixed with 2% KMnO₄ (45 min at room temperature), dehydrated in a graded ethanol series, transferred to propylene oxide, and infiltrated in Epon/Araldite which was polymerized at 60°C. The electron microscopy specimens were sectioned (70 nm thickness) and mounted on copper 200 mesh formvar coated grids. The grids were stained for 30 min with aqueous 2% uranyl acetate with Triton X-100, and then stained with Reynold's lead citrate for 10 min. Grids were imaged with a Philips 410LS transmission electron microscope at 80 kV with 3,000 and 24,000 times magnification.

CELL CULTURE AND TRANSIENT TRANSFECTION

Human embryonic kidney 293 cells stably expressing the Epstein-Barr virus nuclear antigen (293E) cells were maintained in low-

calcium-serum-free medium (LC-SFM) supplemented with 1% BCS and 50 μ g/ml G418 as previously described [Shi et al., 2005]. Cells were maintained in agitated (110–130 rpm) shaker flasks under standard humidified conditions (37°C and 5% CO₂). One day prior to transfection, cells were diluted to a density of 0.25 \times 10⁶/ml in LC-SFM growth medium supplemented with 1% BCS and transfected 18–24 h later when the density reached approximately 0.5 \times 10⁶ cells/ml. Plasmid DNA and polyethylenimine (PEI) were mixed together in LC-SFM medium at 1 μ g/ml and 2 μ g/ml of culture to be transfected, respectively. Upon the addition of PEI to the DNA, the mixture was vortexed, incubated for 15 min and added to the cells. Cells were harvested 96 h post-transfection by centrifugation at 2,000 rpm for 10 min, followed by a wash with ice cold phosphate-buffered saline (PBS) and a subsequent centrifugation step at 3,000 rpm for 15 min. The cell pellets were frozen at –80°C.

CYSTEINE ACCESSIBILITY ASSAY

Cysless Ste2p mutants were overexpressed in 293E cells. Pelleted cells were lysed by French press at 1,500 psi, then centrifuged at 4,000 rpm to remove debris. The remaining suspension was centrifuged at 100,000g for 60 min. The final membrane pellet was resuspended in 10 ml buffer (50 mM PBS, pH 8.0, 100 mM NaCl, 5 mM MgCl₂). Fluorescein-5-maleimide (final concentration 0.5 mM) was added to 5 ml of the resulting resuspended membrane fraction with or without 1 μ M α -factor. After 30 min incubation at 4°C, the reaction was stopped by the addition of 5 mM β -mercaptoethanol. The labeled membrane fraction was then resuspended in buffer (50 mM PBS, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1% DM, 5 mM β -mercaptoethanol), stirred for 1 h and centrifuged at 10,000 rpm. The resulting suspension was then applied to Ni-NTA affinity resin. The column was washed with 10 column volumes of buffer A (50 mM PBS, pH 8.0, 100 mM NaCl, 0.05% DM, 5 mM MgCl₂, 20% glycerol (v/v)) containing 50 mM imidazole and eluted with buffer A containing 250 mM imidazole. The eluted samples were analyzed by SDS-PAGE. The gel was scanned using a STORM 840 instrument (Amersham Biosciences) with excitation/emission wavelengths of 450 nm/520 nm to assess covalent linkage of fluorescein-5-maleimide (FM) to the single Cys sites. After scanning, the same gel was stained by Coomassie blue for direct detection of Cysless, Cysless-I24C and Cysless-I29C loading.

PROTEIN ANALYSIS

Protein was analyzed by SDS-PAGE on 8% acrylamide gels, stained with Coomassie Brilliant Blue R-250 or transferred to PVDF membrane for western blot. Purity of protein samples was determined by visual inspection of Coomassie stained gels. The concentration of purified proteins was measured either by the Bio-Rad BCA protein assay or by visual inspection of coomassie stained gel bands using purified glutathione-S-transferase (GST) as a standard. The concentration of the GST standard was measured by adsorption based upon the assumption that 0.5 mg/ml protein has an OD_{280 nm} of 1.

RESULTS

AMINO ACID SEQUENCE ALIGNMENT AND SECONDARY STRUCTURE PREDICTIONS FOR THE N-TERMINAL REGION OF Ste2p

A mating functionality for the N-terminal domain of Ste2p was recently proposed based on characterization of a Δ N20 mutation of the receptor's extracellular N-terminal tail [Shi et al., 2007]. In order to examine conservation of this domain in other fungi, the N-terminal regions of *S. cerevisiae* Ste2p (SC Ste2p), *Lachancea kluyveri* Ste2p (LK Ste2p), *Candida albicans* Ste2p (CA Ste2p), and *Schizosaccharomyces pombe* Ste2p (SP Ste2p) were compared by amino acid sequence alignment. TMHMM-2.0 was used to predict N-terminal regions for alignment by T-Coffee (Fig. 1 and Supplemental Fig. 1A) [Notredame et al., 2000; Kall et al., 2004, 2005]. All four Ste2p receptors were predicted to maintain a 40–50 amino acid long N-terminal domain. Secondary structure analyses of the N-terminus of these proteins by nnPREDICT indicated that all have a conserved putative β strand-loop- β strand fold. Sequence alignment highlighted conservation of Pro 19 and Gly 31, located on opposite ends of the first putative β strand, as well as two hydrophobic Ile residues in the first putative β strand (Fig. 1).

IDENTIFICATION OF SPECIFIC N-TERMINAL AMINO ACIDS INVOLVED IN MEDIATING MATING EVENTS BY TARGETED SCANNING MUTAGENESIS

We used scanning mutagenesis to examine the functionalities of specific residues in the N-terminal region. Residues comprising the putative β strand-loop- β strand fold domain (Thr 23–Ile 36) and a number of additional proximal unique sites such as the potential glycosylation site Asn 46 and the structurally constrained Pro 15 and Pro 19 were mutated. In total, 17 substitution mutants were constructed by hydrophobic/hydrophilic amino acid exchange such that hydrophobic amino acids were mutated to hydrophilic Cys, and hydrophilic amino acids were mutated to hydrophobic Ala (Fig. 2A).

Cell growth arrest assays of the receptor-null MATa strain expressing Ste2p point mutants indicated that all but four mutants maintained α -factor mediated G1 arrest activity comparable to the same strain overexpressing wild-type Ste2p (Fig. 2B). The four mutants deficient in G1 arrest (P19C, Y26C, G31A, and N46A) were also found to have reduced mating efficiency (Fig. 2C,D). This loss of G1 arrest signaling functionality could be equally well due to negligible overexpression levels of the mutant receptor as to effect of mutation on receptor functionality. As such these four mutants

are not further pursued in this work. Eight of the remaining mutant strains (T23A, T27A, S28A, Y30C, G33A, S34A, T35A, and I36C) maintained both G1 arrest (serving as an indication of good receptor overexpression) and mating activities comparable to wild-type. Two glycosylation site mutant strains, N25A and N32A, maintained strong signaling but showed minor decreases in mating efficiency (32% and 49% of the wild-type expressing strain respectively). Interestingly, receptor-null MATa strains overexpressing Ste2p with P15C, I24C, or I29C mutations demonstrated wild-type G1 arrest (Fig. 2B) as well as wild-type like budding index and mating projection phenotypes (Supplemental Fig. 2), but were almost sterile in the mating assay (Fig. 2C). Quantitation indicated only 5.6% (P15C), <0.9% (I24C), and 4.6% (I29C) mating efficiency compared to the same receptor-null strain expressing wild-type Ste2p (Fig. 2D).

MUTATIONAL ANALYSES OF Ile 24 AND Ile 29 REVEAL THE IMPORTANCE OF THE HYDROPHOBIC NATURE OF THESE SITES TO MATING ACTIVITY

Both Ile 24 and Ile 29 residues are located in the first putative β -strand of the predicted β strand-loop- β strand structure in the N-terminus of Ste2p (Fig. 1). These were replaced with hydrophobic (Ala) and polar (Cys) residues to assess the mode of contribution of the residues to functionality.

Cell growth arrest assays demonstrated that receptor null MATa *ste2* Δ strains overexpressing Ste2p mutants encoding I24C, I24A, I29C, or I29A maintained similar halo size to the wild-type expressing strain (Fig. 3A). Western blot analyses showed that the mutant strains produced comparable levels of receptor to wild-type Ste2p. The growth arrest phenotypes of these mutants were further assessed by budding index (Supplemental Fig. 2A) and mating projection formation (Supplemental Fig. 2B) assays. These mutant strains all resembled wild-type at this level. FUS1-LacZ inductions were decreased slightly for I24C (50.8%), I24A (91.7%), and I29A (67.4%) after a 2 h treatment with α -factor, whereas mutation of Ile 29 to Cys led to a slight increase (108.4%) compared to wild-type (Fig. 3B). Finally, the mutant strains had wild-type-like morphologies (Fig. 3C) and accumulated Calcofluor binding material (chitin) in the cell projection (Supplemental Fig. 3) after α -factor was introduced.

Both I24C and I29C caused virtually sterile phenotypes (Fig. 4A). In contrast both I24A and I29A, which maintain the hydrophobic natures of the side chains, but reduce the overall size of the residues,

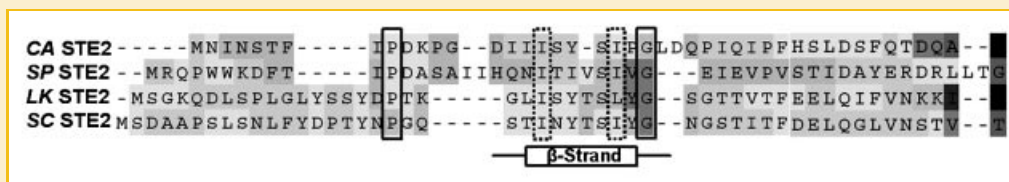


Fig. 1. Sequence alignments of yeast pheromone α -factor receptors from different organisms. The N-terminal regions of *Saccharomyces cerevisiae* Ste2p (SC Ste2p), *Lachancea kluyveri* Ste2p (LK Ste2p), *Candida albicans* Ste2p (CA Ste2p) and *Schizosaccharomyces pombe* Ste2p (SP Ste2p) were identified using the TMHMM algorithm [Kall et al., 2004, 2005]. The secondary structures of these receptors were calculated by nnPREDICT and aligned using T-Coffee [Kneller et al., 1990; Notredame et al., 2000]. The boxes highlight conserved residues.

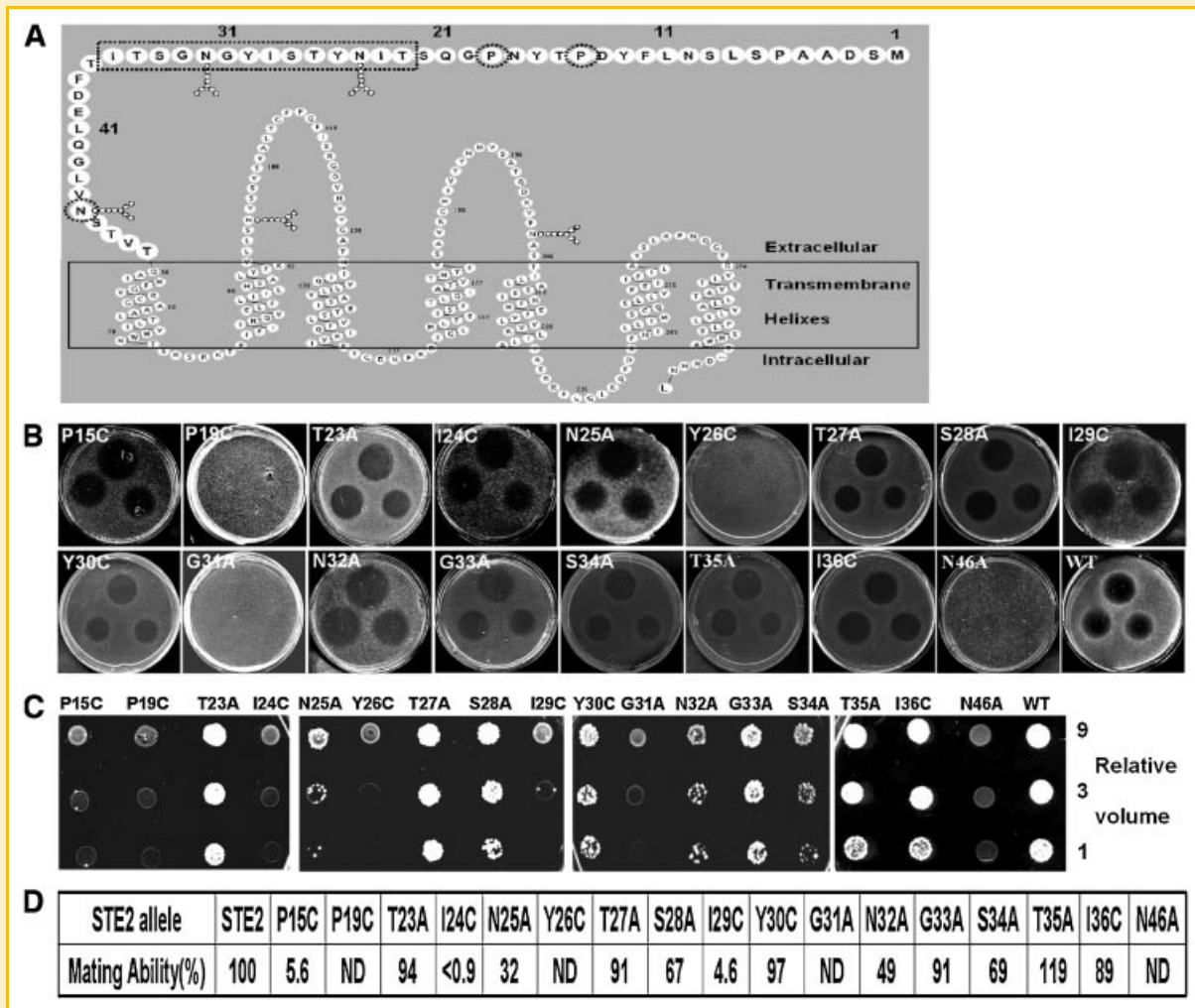


Fig. 2. Scanning mutagenesis of targeted regions of the N-terminal domain of Ste2p. A: Topology structure of Ste2p. Amino acid residues are indicated by their one-letter code and are displayed in the predicted positions of the seven transmembrane domains. Residues targeted for mutagenesis are indicated in dashed circles or the rectangle. B: Halo assay determination of α -factor stimulated signaling and cell cycle arrest activity in a receptor null *MATa ste2 Δ* yeast strain overexpressing Ste2p N-terminal point mutants. Two, 1, and 0.5 μ l volumes of a 10 mM α -factor stock solution were spotted in positions upper, lower left and lower right respectively. C: Auxotrophic MAT α was mated with receptor null *MATa ste2 Δ* yeast strains expressing Ste2p N-terminal point mutants. D: Quantitative mating efficiencies for receptor null *MATa ste2 Δ* yeast strains expressing Ste2p N-terminal point mutants determined by the method of Sprague [1991]. Wild-type strain mating ability was considered to be 100%.

yielded strains that mated, but with lower efficiency than wild-type (Fig. 4A,B). Further to this, DAPI staining was used to assess mating pair assembly and differentiate prezygote (two distinct nuclei) from zygote (one fused nucleus) formation. Comparing the total number of assembled pairs (prezygote + zygote) as a percentage of MATa cells shows a general decrease in mating pair assembly for the mutants compared to WT (25.4% for WT and 6.8%, 9.1%, 9.6%, and 11.1% for I24C, I24A, I29C, and I29A, respectively). Analysis of the relative accumulation of prezygotes to zygotes emphasizes that the observed decrease in mating pair assembly is primarily a reflection of reduced relative zygote accumulation, while relative levels of arrested prezygotes actually increased for the Ile to Cys mutants (Fig. 4C). Specifically, overexpression of the polar Ste2p mutants (I24C and I29C) led to 1.3- and 1.9-fold increases in prezygote accumulation, and 16.5- and 7.1-fold decreases in zygote

accumulation compared to the wild-type Ste2p expressing strain. In contrast, strains expressing mutations to Ala (I24A and I29A), caused only 3.2- and 1.6-fold decreases in zygote accumulation, and 0.5- and <0.1-fold increases in prezygote accumulation respectively. In light of the dramatic observed decrease in mating efficiency for I24C (0.9%), the % zygote accumulation (1.3%) is still fairly high. This discrepancy is most likely explained by mis-assigned zygotes in instances where nuclei were too close to be distinguished as unfused. However the overall impact of any such mis-assignment would be to reduce the apparent prezygotic accumulation levels below actual values, further emphasizing the significance of the observed effects.

Electron microscopic (EM) examination of the arrested prezygotes for I24A and I29C strains showed evidence of large regions of apposition between the two mating cells, suggesting the prezygote

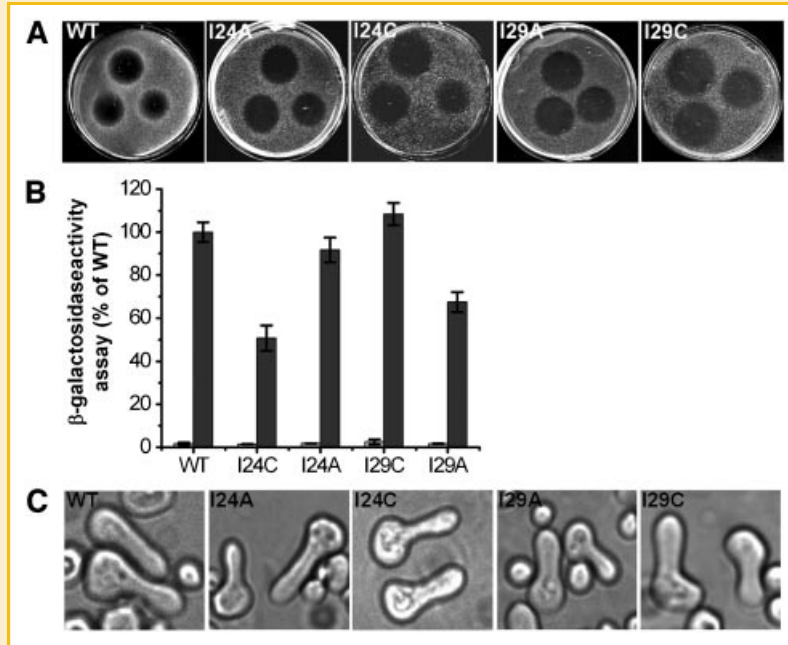


Fig. 3. Characterization of the signaling functionalities of Ile 24 and Ile 29 Ste2p mutants overexpressed in receptor null strains. A: Halo assay determination of α -factor stimulated signaling and cell cycle arrest activity in a receptor null *MATa ste2 Δ* yeast strain overexpressing Ste2p, or mutants thereof I24A, I24C, I29C, and I29A. Two, 1, and 0.5 μ l volumes of a 10 mM α -factor stock solution were spotted in positions upper, lower left and lower right respectively. B: FUS1 promoter activation. β -galactosidase activity from a FUS1-lacZ fusion (plasmid pSB231) in a receptor null *MATa ste2 Δ* yeast strain overexpressing Ste2p, Ste2pl24A, Ste2pl24C, Ste2pl29C, and Ste2pl29A with (dark bar) or without (light bar) a total of 1.5 μ M α -factor for 2 h. The β -galactosidase activity for wild-type strain was normalized to 100%. C: Morphology of receptor null *MATa ste2 Δ* yeast strain overexpressing Ste2p, or mutants thereof I24A, I24C, I29C, and I29A 6 h after the addition of 5 μ M α -factor.

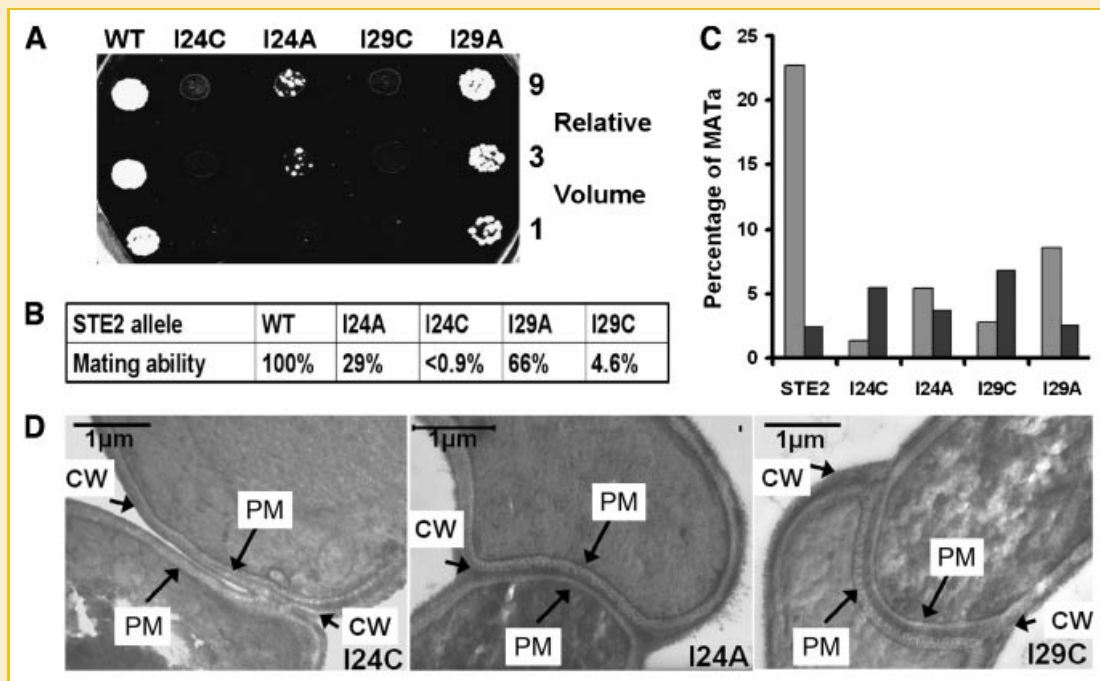


Fig. 4. Characterization of the mating abilities of Ile 24 and Ile 29 Ste2p mutants over expressed in receptor null strains. A, Auxotrophic *MATa* was mated with receptor null *MATa ste2 Δ* yeast strains expressing Ste2p, or mutants thereof I24A, I24C, I29C, and I29A respectively. B: Quantitative mating efficiency of receptor null *MATa ste2 Δ* yeast strains expressing Ste2p N-terminal point mutants by the method of Sprague. C: Comparison of the accumulation of prezygotes (dark gray) and zygotes (light gray) assessed by observation of nuclear fusion in DAPI labeled cells. D: Representative mating deficient I24C, I24A, and I29C arrested prezygotes imaged by electron microscopy. Cell wall (CW), plasma membrane (PM). Bars are 1 μ m.

has been arrested for a significant amount of time prior to fixation (Fig. 4D). Asymmetric cell wall thinning at the fusion site was similar to that observed for the Ste2p Δ N20 truncation [Shi et al., 2007]. For strains expressing the I24C Ste2p mutant, prezygotes arrested following cellular apposition, but showing only partial fusion/remodeling of the cell wall (Fig. 4D). This reduced cell wall fusion defect is likely a significant contributor to the particularly low occurrence of mating pair assembly (6.8%) observed for this mutant.

Ile 24 AND Ile 29 ARE SOLVENT ACCESSIBLE IN ACTIVATED AND RESTING RECEPTOR STATES

Native Ste2p contains two potentially reactive Cys residues, Cys 59 and Cys 252. These were simultaneously mutated to serine to produce a "Cysless" Ste2p that does not react with disulfides. In this background, Ile 24 and Ile 29 were individually mutated to Cys, producing single cysteine Ste2p mutants Cysless-I24C and Cysless-I29C. Growth arrest assays indicated that receptor null strains overexpressing Cysless, Cysless-I24C and Cysless-I29C maintained similar signaling to the same strain overexpressing wild-type receptor (Fig. 5A). Mating of the Cysless expressing mutant strain

with a MAT α partner showed some reduction compared to wild-type. In contrast, Cysless-I24C and Cysless-I29C expressing mutant strains lost almost all mating ability (Fig. 5B). These results are consistent with previous reports for Cysless Ste2p [Lin et al., 2003, 2004; Choi and Konopka, 2006; Hauser et al., 2007] and the I24C and I29C mutants described above.

The solvent accessibility of the Cys residues in Cysless-I24C and Cysless-I29C were assessed by reaction with fluorescein-5-maleimide (FM), a well characterized sulfhydryl reagent that reacts with cysteines that are located in solvent accessible environments [Poelarends and Konings, 2002; Dastidar et al., 2007]. The chemical reactions were carried out on native 293E membranes presenting either Cysless-I24C-His or Cysless-I29C-His, where His is a C-terminal 8 \times histidine tag for purification purposes. Ste2p expressed in mammalian cells has been shown to be predominantly localized to plasma membranes and to maintain proper folding and ligand binding functionality therein [Shi et al., 2005].

The accessibility assay was initiated by treating membrane fractions with FM. Subsequently the membranes were solubilized in detergent buffer, and the mutant receptors enriched by Ni-NTA affinity chromatography. These were then visualized by fluorescent detection following SDS-PAGE to assess covalent linkage of FM to the single Cys sites. Both Cysless-I24C and Cysless-I29C yielded bright fluorescent bands around 55 kDa (the molecular weight of fully glycosylated recombinant Ste2p [Shi et al., 2005]) in both the presence and the absence of ligand. Thus, the Ile 24 and Ile 29 sites appear to be solvent accessible in both resting and activated receptor states (Fig. 5C upper panel). In contrast, Cysless Ste2p was not labeled under identical conditions, highlighting the specificity of the reaction of FM with the single cysteine sites (Fig. 5C upper panel). Coomassie blue staining of the same gel is presented as a control for sample loading onto the gel (Fig. 5C bottom panel).

DISCUSSION

S. cerevisiae mating is a multi-step process including: induction of the pheromone response leading to G1 arrest, cell contact with a partner of opposite mating type, cell wall remodeling, plasma membrane fusion, karyogamy, and zygote vegetative growth [White and Rose, 2001; Elion et al., 2005]. The mating type specific Ste2p and Ste3p receptors are known to mediate the initial pheromone response leading to G1 arrest, through well characterized effector signal transduction pathways [Bardwell, 2005; Elion et al., 2005; Slessareva and Dohlman, 2006]. However, mechanisms modulating the mating steps following G1 arrest remain, for the most part, enigmatic. Deletion analyses of a number of other yeast proteins including Aga1p, Fig2p, Fus1p, Fus2p, and Prm1p, highlight some of the other protein machinery involved in mediating cell contact, cell wall degradation and membrane fusion/pore formation events during mating [Elion et al., 1995; Gammie et al., 1998; Heiman and Walter, 2000; Zhang et al., 2002; Huang et al., 2003; Nolan et al., 2006; Jin et al., 2008]. Membrane localized ergosterol has also been shown to be involved in the promotion of signaling and membrane fusion events [Jin et al., 2008].

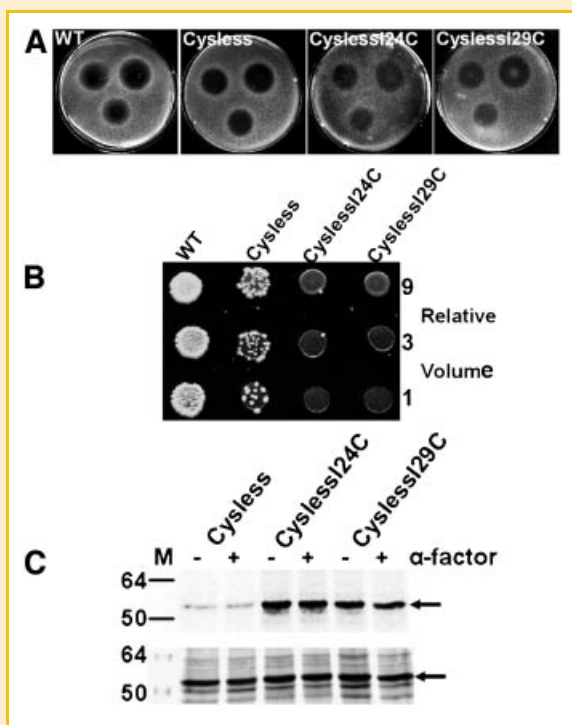


Fig. 5. Solvent accessibility of residues 24 and 29 in the N-terminal tail of Ste2p. A: Halo assay determination of α -factor stimulated signaling and cell cycle arrest activity in a receptor null MAT α Ste2 Δ yeast strain overexpressing Ste2p N-terminal mutants. Two, 1, and 0.5 μ l volumes of a 10 mM α -factor stock solution were spotted in positions upper, lower left and lower right respectively. B: Auxotrophic MAT α was mated with receptor null MAT α ste2 Δ yeast strains expressing Ste2p, Cysless Ste2p, Cysless-I24C and Cysless-I29C mutants. C: Cysteine accessibility of residues Ile 24 and Ile 29 by reaction with fluorescein-5-maleimide. The upper panel was scanned with excitation/emission wavelength of 450 nm/520 nm to show emission at 520 nm; the bottom panel was the same gel stained by Coomassie blue.

Evidence also suggests the pheromone receptors themselves may mediate other steps in the mating process. Specifically, Ste2p has been implicated in mating partner discrimination [Jackson et al., 1991] and more recently as a possible modulator of mating efficiency at the level of cell wall degradation [Shi et al., 2007]. Identification of this latter putative role was based on characterization of a Δ N20 truncation of Ste2p. This truncation reduced mating efficiency, while maintaining wild-type like G1 arrest signaling. EM analyses highlighted mating arrest at a prezygotic stage after cell contact, during cell wall remodeling and degradation. The Ste2p N-terminal tail was also implicated in modulation of an interaction between activated Ste2p and Ste3p receptors and associated lipid mixing functionalities in vitro [Shi et al., 2007]. However, the *ste2- Δ N20* mutant did not have a discernable membrane fusion defect in vivo. The concept of a Ste2p/Ste3p interaction mediating lipid mixing/mating functionalities has not been pursued at this time due primarily to a recent and ongoing shortage of commercially available a-factor, an essential component of in vitro Ste3p functional assays. Rather a systematic mutational screening of regions of the N-terminal domain of Ste2p has been completed to gain further insight into mechanisms associated with mating functionalities of this receptor. Scanning mutagenesis of N-terminal residues Thr 23 through Ile 36, plus Pro 15, Pro 19, and Asn 46 (Figs. 1 and 2) was used to assess contributions to receptor activities. This targeted region includes the N-glycosylation sites Asn 25 and Asn 32. Effects of mutations were characterized in vivo upon expression of mutant receptors in a receptor null yeast strain.

Of the 17 mutations, eight (T23A, T27A, S28A, Y30C, G33A, S34A, T35A, and I36C) had no effect on mating. In contrast P19C, Y26C, G31A, and N46A led to loss of both G1 arrest and mating when expressed in receptor null strains. This result for N46A is at odds with a previous report where the mutation of N46Q maintained wild-type-like signaling characteristics [Mentesana and Konopka, 2001]. Asn 46 is a site that maintains the glycosylation sequence motif (Asn-X-Ser/Thr), but is not in fact glycosylated. One interpretation of this results is that mutation to a small hydrophobic residue affects micro-domain structure similar to what was previously observed for N46C [Lin et al., 2004]. However an alternative possibility is that the mutation leads to a significant decrease in overexpression levels. Mutation of demonstrated N-glycosylation sites, N25A and N32A, led to minor decreases in mating efficiencies, but maintained normal G1 arrest. These latter results are consistent with previous mutations at these sites [Mentesana and Konopka, 2001] and suggest a potential link between glycosylation and mating. However, mutation of Thr 27 and Ser 34, sites which respectively comprise part of the glycosylation sequence motif for Asn 25 and Asn 32, are known to diminished receptor glycosylation [Mentesana and Konopka, 2001]. Thus the lack of effect of the T27A and S34A mutations on either G1 arrest or mating as observed herein, implies that glycosylation structures are not important for mating.

Mutation at three other sites including, P15C, I24C, and I29C (Figs. 1 and 2), led to wild-type like receptor mediated G1 arrest but significant loss of mating efficiency. This phenotype is similar to that reported previously for the Δ N20 mutation [Shi et al., 2007]. The amino acid side chain of Pro has a constrained structure related

to its ring that restricts phi to -60° . This feature lends itself to mediating important roles in protein conformation/orientation such as helix “breaks” or in the formation of turn structures. Thus mutations P15C and P19C likely have an impact on the orientation/structure of the full length N-terminal domain and suggest a similar structural mechanism may modulate the Δ N20 mutant phenotype observed previously. However, unlike P19C, P15C affected mating efficiency only and not G1 cell cycle arrest. This difference highlights the extent of subtleties that are likely involved in receptor mediated yeast mating functionalities. The observed G1-arrest and loss of mating efficiency resulting from mutation of the conserved Ile 24 and Ile 29 residues, implies that the presence of accessible hydrophobic side chains at these sites is important in mediation of Ste2p mating efficiency. Further to this, in the case of Ile 24, it may not only be the hydrophobic nature, but also the specific size and shape of the hydrophobic side chain. The most likely role for these three residues lies in mediation of some form of interaction. Pro 15 would potentially be contributing to the overall conformational/orientational presentation of the region, with Ile’s 24 and 29 contributing to the formation of a unique surface for specific interaction. Speculatively, such an interaction could be with other extracellular loop regions of Ste2p, the pheromone ligand or other proteins. Indeed, previous work in our laboratory has demonstrated that the N-terminal region of Ste2p mediates an interaction with Ste3p in vitro [Shi et al., 2007]. Whether with Ste3p, ligand or some other protein entity, such an interaction could potentially modulate receptor signaling toward activation of novel effector signal transduction pathways, as previously proposed for polarity and orientation establishment [Butty et al., 1998; Nern and Arkowitz, 1999].

EM results for I24C, I24A, I29C, and I29A show arrest of prezygotes after cellular apposition and some cell wall remodeling, but prior to or during cell wall degradation. This phenotype is similar to those observed for the Δ N20 mutant as well as in the cases of *fus1*, *fus2*, and *rvs161* mutants [Gammie et al., 1998; Shi et al., 2007]. Proteins encoded by these latter three genes have been implicated in cell wall degradation activities. The arrested cell wall degradation phenotype is also similar to that observed in mutants where pheromone biosynthesis was reduced, supporting a model in which higher pheromone concentrations are needed to mediate mating events at the level of cell contact and cell wall degradation [Brizzio et al., 1996]. A requirement for higher pheromone concentrations during mating potentially correlates with our proposed role for the N-terminal domain of Ste2p in later stages of mating. An N-terminally modulated lower affinity pheromone binding site, that is activated at higher pheromone concentrations leading to modulation of different effector signaling could account for our observations. However other possible mechanisms cannot be discounted at this time. Transactivation type signaling mediated by the N-terminal domain would also fit the current results as has been proposed previously [White and Rose, 2001; Shi et al., 2007]. In this model an interaction between Ste2p and another molecule presented on the surface of the opposite mating type apposed cell would lead to alternate effector signaling. It is even possible that, low affinity ligand binding and transactivation are both required to mediate the signaling. Finally, mislocalization of the mutagenized receptor

within the plasma membrane could also be a factor. This last possibility might explain the partial reduction in prezygote formation observed for some mutants (e.g., I24A and I29A), an event which is dependent upon proper receptor localization [Jackson et al., 1991]. Ultimately further experiments are needed to distinguish between possible mechanisms.

Overall our results emphasize specific functionalities in mating for residues of the N-terminal domain of the Ste2p receptor and highlight the extent of subtleties likely involved in mediation of receptor activities. We speculate that residues mediating mating efficiency but not G1 arrest support novel receptor interactions, leading to stimulation of alternate signaling effector pathways.

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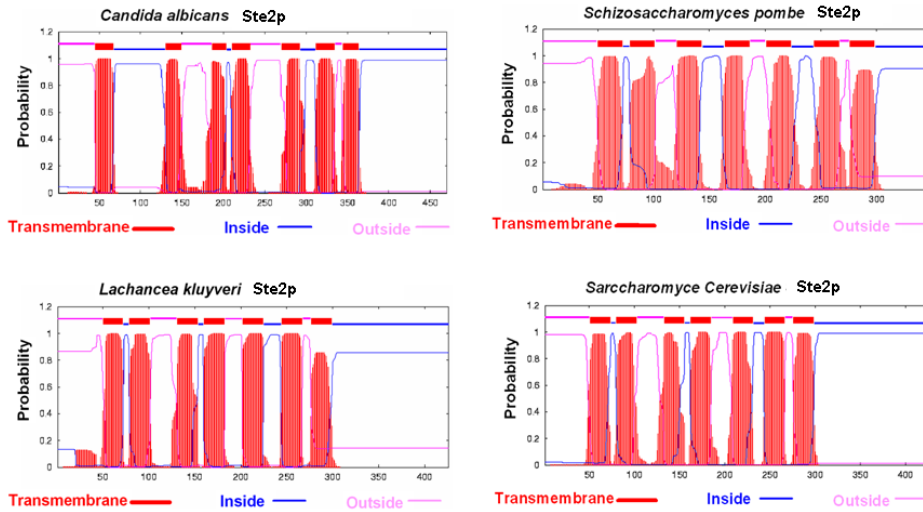
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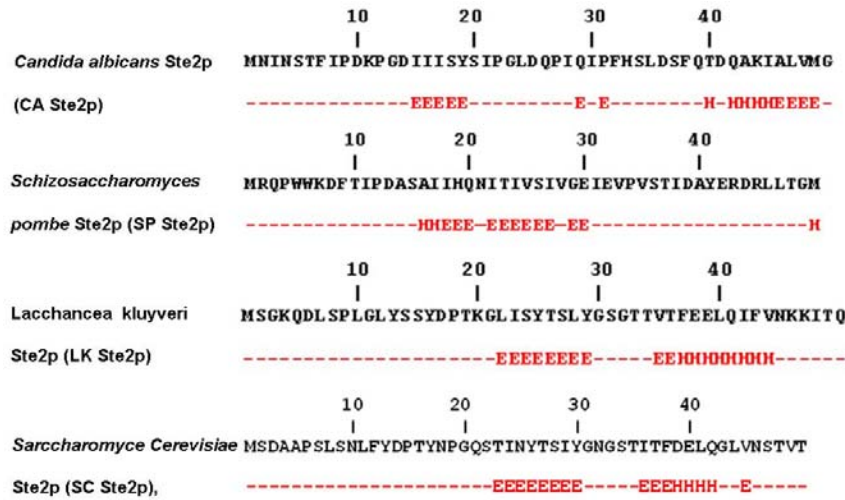
Supplemental Table 1. The primers used in the scanning mutagenesis experiments.

Allele	Forward	Reverse
P15C	CTCCTTCATTGAGCAATCTATTTTATGATTGCACGTATAATC CTGGTCAAAGCACCATTAAAC	TTAATGGTGCTTGACCAGGATTATACGTGCAATCATAAAATAGATTGCT CAATGAAGGAG
P19C	GCAATCTATTTTATGATCCAACGTATAATTGTGGTCAAAGC ACCATTAACTACACTTCC	GGAAGTGTAGTTAATGGTGCTTGACCACAATTATACGTTGGATCATAAA ATAGATTGC
T23A	CCAACGTATAATCCTGGTCAAAGCCCCATTAACACACTTCC CATATATG	CATATATGGAAGTGTAGTTAATGGGGCTTGACCAGGATTATACGTTGG
I24A	CGTATAATCCTGGTCAAAGCACCGCTAACTACACTTCCATA TATGGG	CCC ATA TAT GGA AGT GTA GTT AGC GGT GCT TTG ACC AGG ATT ATA CG
I24C	CGTATAATCCTGGTCAAAGCACCTGTAACACTACACTTCCATA TATGGG	CCCATATATGGAAGTGTAGTTACAGGTGCTTGACCAGGATTATACG
N25A	CCTGGTCAAAGCACCATTGCTACACTTCCATATATGGG	CCCATATATGGAAGTGTAGGCAATGGTGCTTTGACCAGG
Y26C	CCTGGTCAAAGCACCATTAACTGTACTTCCATATATGGGAA TGGATC	GATCCATTCCCATATATGGAAGTACAGTTAATGGTGCTTTGACCAGG
T27A	CTGGTCAAAGCACCATTAACTACGCTCCATATATGGGAAT GGATCTAC	GTAGATCCATTCCCATATATGGAGGCGTAGTTAATGGTGCTTTGACCAG
S28A	CAAAGCACCATTAACTACACTGCCATATATGGGAATGGATC TAC	GTAGATCCATTCCCATATATGGCAGTGTAGTTAATGGTGCTTTG
I29A	CAAAGCACCATTAACACTACACTTCCGCTTATGGGAATGGATC TACCATCAC	GTG ATG GTA GAT CCA TTC CCA TAA GCG GAA GTG TAG TTA ATG GTG CTT TG
I29C	CAAAGCACCATTAACACTACACTTCTGTTATGGGAATGGATC TACCATCAC	GTGATGGTAGATCCATTCCATAACAGGAAGTGTAGTTAATGGTGCTTTG
Y30C	CCATTAACTACACTTCCATATGTGGGAATGGATCTACCATC AC	GTGATGGTAGATCCATTCCCATATGGAAGTGTAGTTAATGG
G31A	CACCATTAACTACACTTCCATATATGCCAATGGATCTACCA TCACCTTCGATGAG	CTCATCGAAAAGTGATG GTAGATCCATTGGCATATATGGAAGTGTAGTTA ATGGTG
N32A	CTACACTTCCATATATGGGGCTGGATCTACCATCACTTTCCG	CGAAAAGTGATGGTAGATCCAGCCCATATATGGAAGTGTAG
G33A	CTACACTTCCATATATGGGAATGCTCTACCATCACTTTCCG ATGAGTTG	CAACTCATCGAAAAGTGATG GTAGAGGCATTCCCATATATGGAAGTGTAG
S34A	CACTTCCATATATGGGAATGGAGCCACCATCACTTTCCGATG AGTTGC	GCAAATCATCGAAAAGTGATGGTG GCTCCATTCCCATATATGGAAGTG
T35A	CCATATATGGGAATGGATCTGCCATCACTTTCCGATGAGTTG	CAACTCATCGAAAAGTGATGGCAGATCCATTCCCATATATGG
I36C	CTTCCATATATGGGAATGGATCTACCTGTACTTTCCGATGAG TTGCAAGGTTTAG	CTAAACCTTGCAACTCATCGAAAAGTACAGGTAGATCCATTCCCATATATG GAAG
N46A	GAGTTGCAAGGTTTAGTTGCCAGTACTGTTACTCAGGCC	GGCCTGAGTAAACGACTGGCAACTAAACCTTGCAACTC
C59S	GGCCATTATGTTGGTGTAGATCTGGTGCAAGCTGCTTTG	CAAAGCAGCTGCACCAGATCTGACACCAACATAATGGCC
C252S	CTCATAATGTATCCCAATCTTTGTTGGTTCC	GGAACCAACAAGATTGGGATGACATTATGAG

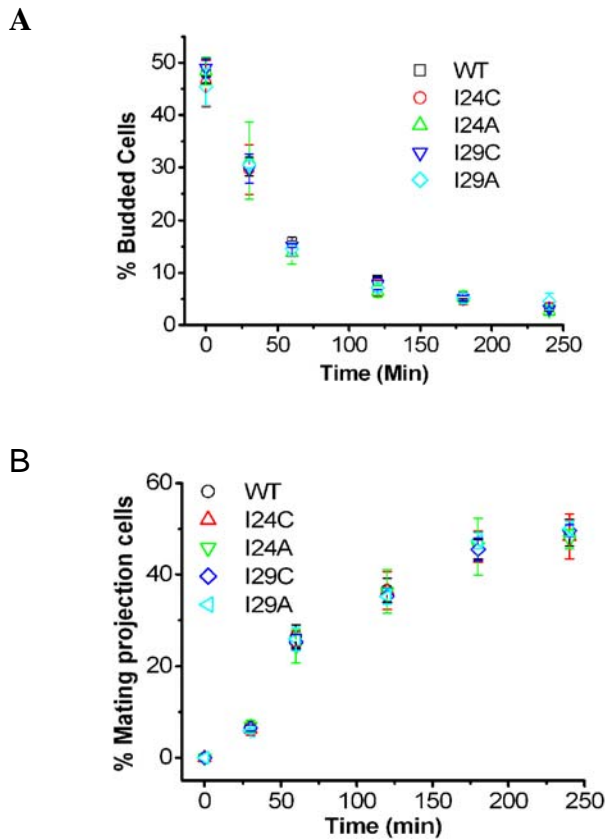
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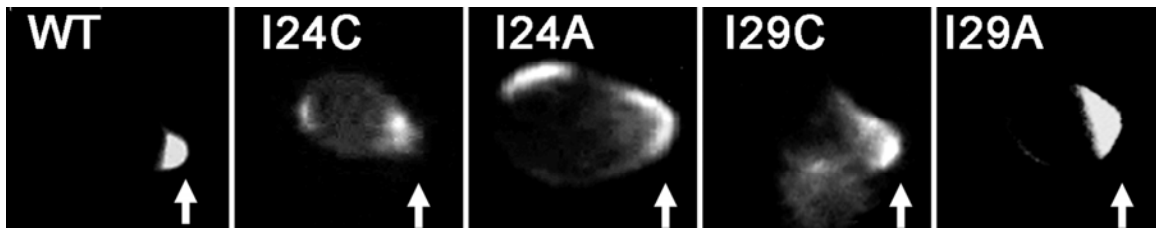
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Supplemental Figure 1. The topology and N-terminal secondary structure predictions for various α -factor mating receptors. A: Topological calculations for *Saccharomyces cerevisiae* Ste2p (SC Ste2p), *Lachancea kluyveri* Ste2p (LK Ste2p), *Candida albicans* Ste2p (CA Ste2p) and *Schizosaccharomyces pombe* Ste2p (SP Ste2p) by TMHMM. (Kall *et al.*, 2004, , 2005) B: Secondary structure calculations for the predicted N-terminal regions of these receptors by nnPREDICT (Kneller *et al.*, 1990).



Supplemental Figure 2. Pheromone cell cycle arrest (budding index) and mating projection formation assays. A: Receptor null *MATa* yeast strain overexpressing either Ste2p wild type (WT) or the indicated Ste2p mutants (I24C, I24A, I29C, I29A) were treated with 1 μ M α -factor. Cell cycle arrest was assessed as the percentage of unbudded, G1-arrested cells. B: Kinetics of mating projection formation by receptor null *MATa* yeast strain overexpressing either Ste2p wild type (WT) or the indicated Ste2p mutants (I24C, I24A, I29C, I29A) after samples were treated with 1 μ M α -factor. The percentage of mating projection formation with respect to total cells was quantified microscopically.



Supplemental Figure 3. Accumulation of Calcofluor binding material (chitin) in the projection sites of I24C, I24A, I29C and I29A mutant alleles. The arrows show the projection and accumulation site.

Supplemental References:

Kall, L., Krogh, A., and Sonnhammer, E.L. (2004). A combined transmembrane topology and signal peptide prediction method. *J Mol Biol* *338*, 1027-1036.

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