Mapping of a Yeast G Protein βγ Signaling Interaction

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Manuscript received June 3, 1998
Accepted for publication September 4, 1998

ABSTRACT

The mating pathway of *Saccharomyces cerevisiae* is widely used as a model system for G protein-coupled receptor-mediated signal transduction. Following receptor activation by the binding of mating pheromones, G protein βγ subunits transmit the signal to a MAP kinase cascade, which involves interaction of Gβ (Ste4p) with the MAP kinase scaffold protein Ste5p. Here, we identify residues in Ste4p required for the interaction with Ste5p. These residues define a new signaling interface close to the Ste20p binding site within the Gβγ coiled-coil. Ste4p mutants defective in the Ste5p interaction interact efficiently with Gpa1p (Gα) and Ste18p (Gγ) but cannot function in signal transduction because cells expressing these mutants are sterile. Ste4 L65S is temperature-sensitive for its interaction with Ste5p, and also for signaling. We have identified a Ste5p mutant (L196A) that displays a synthetic interaction defect with Ste4 L65S, providing strong evidence that Ste4p and Ste5p interact directly *in vivo* through an interface that involves hydrophobic residues. The correlation between disruption of the Ste4p-Ste5p interaction and sterility confirms the importance of this interaction in signal transduction. Identification of the Gβγ coiled-coil in Ste5p binding may set a precedent for Gβγ-effector interactions in more complex organisms.

IN the pheromone response of the yeast *Saccharomyces cerevisiae,* binding of the pheromones **a**-factor and α-factor to the receptors Ste3p and Ste2p, respectively, activates a heterotrimeric G protein composed of Gpa1p (Gα), Ste4p (Gβ), and Ste18p (Gγ) subunits (reviewed in Leberer *et al.* 1997). The Gβγ element communicates the signal to a MAP kinase cascade comprising Ste11p (MAPKKK), Ste7p (MAPKK), and Fus3p (MAPK). Fus3p then activates the cyclin-dependent kinase inhibitor Far1p and the transcription factor Ste12p to bring about cell-cycle arrest and new gene expression required for the mating process. Activation of the MAPK cascade by Gβγ requires Ste20p, a member of the PAK family of kinases, which acts upstream of Ste11p in the pathway (Leberer *et al.* 1992a; Ramer and Davis 1993), and has been shown to phosphorylate Ste11p *in vitro* (Wu *et al.* 1995). Ste20p binds to Ste4p specifically in response to pheromone (Leeuw *et al.* 1998), suggesting a mechanism for pheromone-regulated signal transduction from the G protein to the MAPK cascade, although it remains to be proven that Ste20p directly activates Ste11p. Ste20p, Ste11p, and Ste7p also participate in another signal transduction pathway, in which nutrient deprivation leads to invasive and filamentous growth (Roberts and Fink 1994). In this pathway, Ste7p activates a distinct MAPK, Kss1p, leading to transcription from filamentous-response elements (Cook *et al.* 1997; Madhani *et al.* 1997). Ste5p appears to function as a scaffold, forming a complex with Ste11p, Ste7p, and Fus3p that may prevent crosstalk between these two pathways (Choi *et al.* 1994; Kranz *et al.* 1994; Marcus *et al.* 1994). Ste4p associates with Ste5p (Whiteway *et al.* 1995; Inouye *et al.* 1997; Feng *et al.* 1998; Leeuw *et al.* 1998), possibly bringing Ste20p into proximity with Ste11p and serving as a specificity determinant, ensuring that the pheromone signal is directed to Fus3p to activate the correct target genes for mating. Ste5p may also play a more active role in signal transduction: a hyperactive Ste5p mutant can activate the downstream pathway in the absence of pheromone, and can partially suppress sterility phenotypes caused by null alleles of *STE2, STE4,* or *STE18* (Hasson *et al.* 1994); also, fusion of Ste5p to GST allows pheromone-independent signaling (Inouye *et al.* 1997; Feng *et al.* 1998), perhaps due to the facilitated dimerization of Ste5p.

In mammalian cells, multiple roles have been identified for Gβγ subunits (for reviews see Neer 1994; Hamm 1998), including regulation of various isoforms of adenylate cyclase (Tang and Gilman 1991; Federman *et al.* 1992), phospholipase C (Katz *et al.* 1992), inwardly rectifying potassium channels (Wickman *et al.* 1994), voltage-dependent calcium channels (Dewaard *et al.* 1997; Zamponi *et al.* 1997), sodium channels (Ma *et al.* 1997), and β-adrenergic receptor kinase (Pitcher *et al.* 1992). Gβγ subunits have been shown to activate MAP kinase pathways through Ras- and Rac-dependent mechanisms (Crespo *et al.* 1994; Faure *et al.* 1994; Koch *et al.* 1994), and direct binding of Gβ to the Raf kinase has been shown (Pumiglia *et al.* 1995). However, little

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is understood about the mechanism by which Gβγ subunits interact with and activate their effectors.

The crystal structure of the free Gβγ heterodimer of the G protein transducin reveals a distinctive propeller structure constructed of seven blades that derive from WD repeats in the Gβ primary sequence (Sondek *et al.* 1996). The amino terminus of Gβ extends as a coiled-coil with Gγ that packs against one side of the propeller, contacting blades 4 and 5. This structure is little altered when Gβγ is complexed with Gα (Wall *et al.* 1995; Lambright *et al.* 1996), although binding to the negative regulator phosducin leads to small local conformational changes (Gaudet *et al.* 1996). No structural information is available for a Gβγ element complexed with an effector.

Yeast Ste4p and Ste18p share a high degree of homology with mammalian Gβ and Gγ subunits, except that Ste4p contains a 41-amino-acid insertion within the sixth WD repeat (Whiteway *et al.* 1989). Despite this variation, it is likely that the yeast Ste4p/Ste18p complex has close structural and functional similarity to mammalian Gβγ subunits. The Ste4p interaction with Gpa1p and Ste18p can be modeled on mammalian Gαβγ heterotrimers. Ste4p appears to interact with many proteins within the yeast cell; the investigation of these targets may provide insight into the mechanism of Gβγ signaling in more complex organisms. In two-hybrid assays, Ste4p has been shown to interact with the amino terminus of Ste5p (Whiteway *et al.* 1995) and with Akr1p (Kao *et al.* 1996; Pryciak and Hartwell 1996), a protein involved in receptor endocytosis (Givan and Sprague 1997). Interactions have also been observed with Cdc24p (Zhao *et al.* 1995; Nern and Arkowitz 1998), a protein that connects the pheromone response to morphological changes, and with Syg1p (Spain *et al.* 1995), a protein of unknown function that may be involved in desensitization. The nature of these interactions remains undetermined. Dominant negative mutations of Ste4p have identified two functional regions involved in pheromone signaling (Leberer *et al.* 1992b): in the amino terminus, and in blades 2 and 3 of the β-propeller structure. Of these, K55 and D62 in the amino terminus are required for Ste20p binding (Leeuw *et al.* 1998), but the role of the other region is unclear.

Because the association between Ste4p and Ste5p appears to be critical in directing the pheromone signal to the appropriate effector, we have investigated this contact. The Ste4p-Ste5p interaction is dependent on the presence of Ste18p, and the amino-terminal 214 amino acids of Ste5p are sufficient for interaction with Ste4p (Whiteway *et al.* 1995). This region contains homology to a RING-H2 motif, related to the larger family of RING domains (Klug and Schwabe 1995; Bienstock *et al.* 1996; Borden and Freemont 1996), a structural class of zinc finger implicated in protein-protein interactions. The RING-H2 domain of Ste5p is reported to participate in homodimerization during signal transduction (Inouye *et al.* 1997; Feng *et al.* 1998). We identify hydrophobic residues in the amino terminus of Ste4p that participate in an interaction with the RING-H2 domain of Ste5p.

MATERIALS AND METHODS

Plasmids and strains: Yeast strains are described in Table 1. For regulated expression of *GPA1*, we devised a system whereby the promoter of any gene at its chromosomal locus could be replaced by the *MET3* promoter. A *MET3* promoter fragment was obtained by polymerase chain reaction (PCR; Saiki *et al.* 1988) from pHAM8 (Mountain and Korch 1991) template using the primers AAAAAAAGATCTAGATTTTC CAACG and AAAAAAAGATCTGATATCACAACTGTTACG. This was cloned as a filled-in *Bgl*II fragment into the *Eco*RV site of pBB, which is pBluescript KS+ (Stratagene, La Jolla, CA) in which the *Asp*718 site has been destroyed by filling in and a *Bgl*II linker GGAGATCTCC inserted. A 3846-bp *Bam*HI fragment of pDG82 (gift of D. Gietz) containing the *hisG-URA3-hisG* cassette (Alani *et al.* 1987) was filled in and cloned into the *Sma*I site of the resulting construct to create pBUM. An upstream promoter region and a region encoding the first 106 amino acids of Gpa1p were obtained by PCR from pMN10 template (Miyajima *et al.* 1987) using the primer pairs AAAAAAGGATCCTGTTTTACTCGACTCAACG / AAAAAAA GATCTACAAGGAAGAATACGC and AAAAAAGGATCCGA AATAATGGGGTGTACAG / AAAAAAAGATCTTCACAGTCA AGTTGAATACC and cloned as *Bam*HI/*Bgl*II fragments into the *Bam*HI and *Bgl*II sites, respectively, of pBUM to create pMGII. The *Bam*HI/*Bgl*II fragment of pMGII was transformed into W303-1A to create SDY105. The URA3 sequence was lost by selection on 5-fluoro-orotic acid (PCR Inc.). SDY105 was crossed with W303-1BΔste4. The resulting diploid was sporulated to obtain SDY109. To create SDY110, *STE5* promoter and terminator regions were amplified from genomic DNA by PCR using the oligonucleotide pairs AGAGCTCGAGCGGC CGCAAGCTTAGGGTTACCGGCCT / ATGCCCGAATTCCG CTGTATCCTGTATC and GCCTAGATGCGGCCGCTATAT ATAATCCATATGGAG / CCCGGGATCCGAGTATACACTAA ATTTTATGC and cloned as *Xho*I/*Eco*RI and *Not*I/*Bam*HI fragments, respectively, into the polylinker of pBB creating pBB-STE5#5′3′. The *ADE2* gene was derived as a *Bgl*II fragment from pASZ10 (Stotz and Linder 1990) and cloned into the *Bam*HI site of pBB-STE5#5′3′. The *ste5::ADE2* disruption cassette was excised as a *Not*I fragment and transformed into W303-1BΔste4.

The GAD control plasmid was pGAD2F (Chien *et al.* 1991). GAD-Ste4 was encoded on pKB40.1 (Clark *et al.* 1993). LexA-Gpa1 was encoded on pBTM116-GPA1 (Spain *et al.* 1995), a gift of J. Colicelli. LexA-Akr1 was encoded on pBTM-AKR1 (Pryciak and Hartwell 1996), a gift of P. Pryciak. LexA-Ste5 (aa 1–214) was encoded on M276p16 (Whiteway *et al.* 1995). The Ste5 C177S mutation was generated in pRS313GAL-STE5 (described by Whiteway *et al.* 1995) as follows: single-strand DNA was produced in *E. coli* strain CJ236 (*dut*$^-$ *ung*$^-$) and site-directed mutagenesis was performed as described by Kunkel *et al.* (1987) using oligonucleotide AGGCTCGTCACATAAAGTACTAGAAGCATTCAAAAAGG to yield plasmid pCW151. The mutant *STE5* coding sequence was transferred into pBTM116 (Vojtek *et al.* 1993) using the PCR reaction described by Whiteway *et al.* (1995) to create M277p7. pRL126, encoding LexA-Ste18, was created by inserting *STE18*, isolated as a 350-bp *Eco*RI-*Sal*I fragment from pKB24.5 (Clark *et al.* 1993), into pBTM116. The HA-Ste4

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
W303-1A	*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*	R. Rothstein
W303-1B	*MATα ade2 his3 leu2 trp1 ura3 can1*	R. Rothstein
L40a	*MAT***a** *his3 leu2 trp1 LYS2::LexA-HIS3 URA3::LexA-lacZ*	Vojtek *et al.* (1993)
M364-2C	*MAT***a** *his3 leu2 trp1 far1-1 LYS2::LexA-HIS3 URA3::LexA-lacZ*	Leeuw *et al.* (1995)
W303-1BΔste4	W303-1B *ste4Δ::URA*	Whiteway *et al.* (1989)
SDY105	W303-1A *gpa1::URA3/MET3p-GPA1*	This study
SDY109	W303-1B *ste4Δ::URA3 gpa1::URA3/MET3p-GPA1*	This study
SDY110	W303-1B *ste4Δ::URA3 ste5Δ::ADE2*	This study
DC14	*MAT***a***his1*	Clark *et al.* (1993)
DC17	*MATα his1*	Clark *et al.* (1993)

construct was pL55 (Whiteway *et al.* 1995). For expression of *STE5*, the *STE5* upstream and downstream regions from pBB-STE5#5′3′ were excised as an *Xho*I/*Sac*II fragment and cloned into the polylinker of pRS314 (Sikorski and Hieter 1989) to create pRS314-STE5#5′3′. The *STE5* open reading frame was amplified from plasmid p61 (MacKay 1983) template, a gift of Vivian McKay, using primers TGCGGCGATAT GTAGCTTGTT and GATATGCTGCGGCCGCTCTAATGTT CCAAGTAACACAG and transformed into W303-1A with *Bam*HI-cut pRS314-STE5#5′3′ to create pRS314-STE5. Site-directed mutagenesis to create Ste4p and Ste5p mutants was carried out using a Quikchange kit (Stratagene), according to manufacturer's instructions. Oligonucleotides were supplied by Life Technologies. Plasmids were transformed into yeast strains using lithium acetate (Gietz *et al.* 1992).

Media: Cells were grown in media described by Sherman (1991). Plasmid-containing cells were grown selectively for plasmid maintenance in synthetic medium containing appropriate supplements.

Generation of mutants: *STE4* mutations were generated by low-fidelity PCR on the basis of the protocol of Muhlrad *et al.* (1992) using a 100-μl reaction volume containing 1× Taq polymerase buffer (Promega, Madison, WI) adjusted to 4.5 mm $MgCl_2$, 0.2 mm $MnCl_2$, 0.2 mm dATP, 1 mm dCTP, 1 mm dGTP, 1 mm dTTP, 5 units Taq polymerase, 100 ng pKB40.1 template, and 100 pmol each oligonucleotide primers ATAC CACTACAATGGATGATG and CGTTTTAAAACCTAAGAGT CAC, which hybridize to GAL4 AD and terminator sequences within pGAD2F, respectively. The reaction was incubated for 3 min at 94° followed by 30 cycles of 94° 1 min, 45° 1 min, 68° 90 sec with a final extension at 68° for 10 min. The product was purified using Wizard PCR Preps purification kit (Promega, Madison, WI) and resuspended in 50 μl water. A total of 5 μl of the product, plus 0.25 μg *Bam*HI-cut, filled-in, phosphatased pGAD2F, were transformed into L40a yeast pretransformed with pBTM116-GPA1. Colonies were selected on medium lacking histidine supplemented with 10 mm 3-amino-triazole (Sigma, St. Louis), pooled, and grown selectively for 8 hr in liquid culture at 30°. Harvested cells were incubated in 10 ml Zymolyase solution (1.2 m sorbitol, 0.1 m KPO_4 pH 7.5, 0.4 mg/ml Zymolyase (Seikagaku, Rockville, MD) at 37° for 2 hr, incubated in 10 ml P2 Lysis Buffer (QIAGEN Inc., Chatsworth, CA) for 5 min, then 10 ml P3 neutralization buffer (QIAGEN) for 5 min. DNA was purified on a Tip 500 column (QIAGEN) according to manufacturer's instructions, and resuspended in 100 μl 1× TE pH 8.0. A total of 1 μl of the library was transformed into competent SDY109 cells and selected on synthetic medium supplemented with 2 mm methionine. Sequencing of 12 recovered plasmids randomly chosen indicated an average mutation rate of ~2 per 1000 bases.

Plasmid recovery: Yeast transformants were grown selectively in 2 ml synthetic medium to saturation, harvested, and treated with 0.2 ml Zymolyase solution for 2 hr. Subsequent steps were followed as above, except using QiaPrep Spin miniprep kit (QIAGEN) according to manufacturer's instructions. DNA was resuspended in 50 μl water, of which 1 μl was transformed into *E. coli* XL-1Blue competent cells (Stratagene).

Two-hybrid assays: Quantitative β-galactosidase assays were based on the protocol of Harshman *et al.* (1988). Cultures were grown to saturation, cells were harvested and lysed by three freeze-thaw cycles in lysis buffer (0.1 m Tris-Cl pH 7.5, 0.05% Triton X-100). Samples of cell slurry were incubated at 37° in a reaction mixture containing 500 μl Z buffer (Miller 1972), 100 μl 4 mg/ml *o*-nitrophenyl β-d-galactopyranoside (ONPG; Sigma), and 0.04 m β-mercaptoethanol (Sigma). Reactions were stopped with 250 μl 1 m Na_2CO_3 and read spectrophotometrically at 420 nm. For normalization, cell slurries were diluted in water and their optical densities measured at 600 nm. Arbitrary β-galactosidase activity units were calculated using the formula $U = 1000 \times A420/[(A600/W)\,V\,T]$, where W is the volume of cell slurry in the normalization mixture, V is the volume of cell slurry in the reaction, and T is the incubation time of the ONPG reaction, in minutes. Variability due to the moderate toxicity of GAD-Ste4 was greatly reduced by the *far1-1* mutation in strain M364-2C. For blue colony assays, cells were grown on Hybond-N filters (Amersham, Arlington Heights, IL), frozen in liquid nitrogen, and thawed three times, then placed onto filter paper soaked in Z buffer containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-GAL; Sigma) and 0.04 m β-mercaptoethanol. For growth assays, transformants were grown selectively in liquid culture to saturation, then 10 μl applied to selective plates lacking histidine and supplemented with 10 mm 3-amino-triazole (Sigma). Plates were incubated either at 20° or 30°.

Mating assays: SDY109 colonies transformed with GAD-Ste4 derivatives were patched onto selective medium, then replica-plated onto a lawn of the mating tester strains on YPD plates and incubated at 30° for 16–24 hr. Patched SDY110 transformants of pL55 derivatives were grown on selective synthetic medium containing 2% glucose, then replica-plated onto a lawn of the mating tester strains on YPGAL plates and incubated at different temperatures for 16–24 hr. The mated yeast were then replica-plated onto diploid-selective plates and grown at 30° for a further 24–48 hr.

Western blotting: Immunoblotting (Laemmli 1970) was performed on total cell extracts prepared as follows: 10^9 cells, in mid-log phase, were lysed by beating with glass beads in 50 mm Tris pH 8.0 containing Complete protease inhibitors

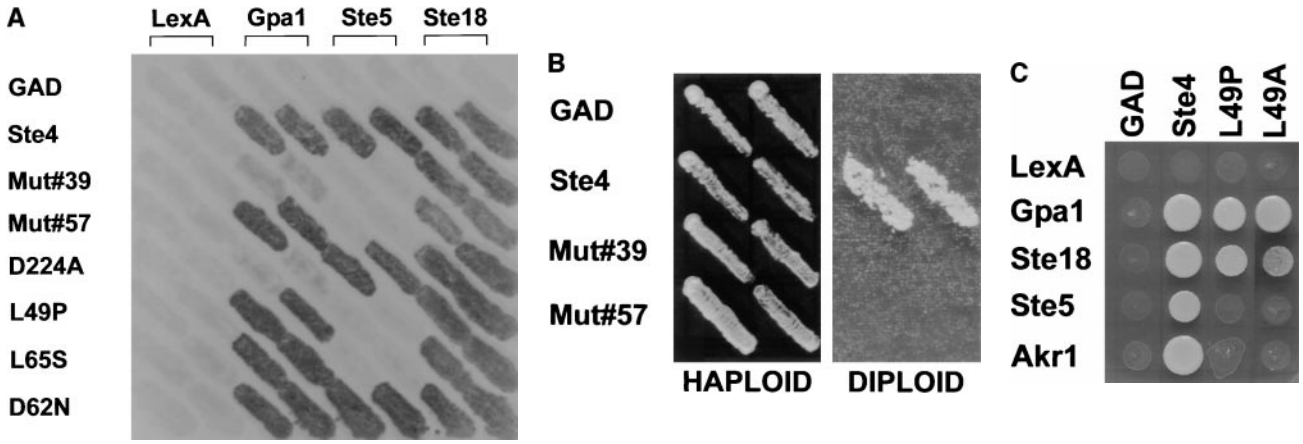

Figure 1.—Interaction assays of Ste4p and mutant Ste4p proteins. (A) Blue colony assay (in strain L40a) for two-hybrid interactions between GAD-Ste4 or GAD-Ste4 mutants, and LexA-Gpa1, LexA-Ste5 (1-214), and LexA-Ste18 baits, as indicated. In each case, two transformants were tested. (B) Mating assay: Haploid strain SDY109 transformants expressing GAD-Ste4 or GAD-Ste4 mutants as the only Ste4p species were incubated with the mating tester strain DC14, and the resulting diploids selected on appropriate minimal medium. (C) Growth assay for two-hybrid interactions between GAD-Ste4 or GAD-Ste4 L49 mutants in combination with the LexA fusions as indicated.

(Boehringer Mannheim, Indianapolis). Supernatants were tested for protein concentration by dot blotting, and extracts from $\sim 10^7$ cells per sample were run on 4–20% SDS polyacrylamide gels (Novex, Encinitas, CA). Proteins were transferred to Hybond ECL membrane (Amersham). HA-tagged Ste4p was probed using 16B12 monoclonal antibody (Babco) and Ste5p was probed using anti-GST-Ste5 polyclonal antiserum (Leeuw *et al.* 1998) and detected using ECL reagents (Amersham). The blots were stripped and reprobed with an anti-Gpa1 polyclonal antibody to control for loading (data not shown).

Structural modeling: The Gpa1p/Ste4p/Ste18p structure was modeled on the coordinates of mammalian Giα1β1γ2 (Wall *et al.* 1995) from the Brookhaven database and realized using RasMol program, developed by Roger Sayle at Glaxo Wellcome.

RESULTS

Creation of a mutant Ste4p two-hybrid library: We set out to define interaction interfaces of Ste4p important in signal transduction by identifying nonsignaling Ste4p mutants and using protein interaction assays to map any defective interactions. In the two-hybrid assay, Ste4p fused to the Gal4p activation domain (GAD-Ste4) interacts strongly with Gpa1p, Ste18p, and Ste5p (amino-terminal residues 1–214) fused to the LexA DNA binding domain (Figure 1A). GAD-Ste4 can also function in the yeast pheromone-response pathway: a strain of the *MAT*α mating-type (SDY109), deleted for chromosomal *STE4*, mated with a *MAT***a** strain (DC14) when transformed with GAD-Ste4 but not when transformed with GAD alone on plasmid pGAD2F (Figure 1B). The GAD-Ste4 transformants did not mate with a *MAT*α strain (DC17; data not shown), indicating that GAD-Ste4 was functioning as part of a receptor-mediated response. Therefore, functional analysis and protein interaction assays can be carried out on the same Ste4p molecule.

A library of mutagenized *STE4* DNA was made by low fidelity PCR from the GAD-Ste4 DNA template (pKB40.1) and cloned into linearized pGAD2F using homologous recombination *in vivo*. The recombination was performed in a two-hybrid test strain (L40a) expressing LexA-Gpa1; GAD-Ste4 fusions that were still able to interact with LexA-Gpa1 were selected. Plasmids were recovered and transformed into strain SDY109, which contains not only a deletion of chromosomal *STE4*, but has *GPA1* under control of the methionine-repressible *MET3* promoter. When this strain is grown in 2 mm methionine, expression of *GPA1* is tightly repressed. In this situation, signaling-competent GAD-Ste4 molecules are free to signal to the downstream pathway, resulting in cell-cycle arrest and allowing the positive selection of nonsignaling GAD-Ste4 mutants.

This series of procedures produced a library enriched for full-length GAD-Ste4 fusions that were defective in signaling.

Ste4p mutants specifically defective in the interaction with Ste5p: Out of 200 SDY109 transformants tested, 64 failed to mate with DC14 at 30°. Plasmids recovered from these were tested in two-hybrid blue colony assays with the LexA fusions. Of the mutant GAD-Ste4 species, 62 showed little or no interaction with LexA-Ste18. These also failed to interact with the LexA-Ste5 construct, which is understandable since Ste18p is required for the Ste4p-Ste5p interaction. Only two mutants, designated 39 and 57, were significantly defective in the LexA-Ste5 interaction while maintaining strong interactions with LexA-Ste18 (Figure 1A). Mutant 39 also had a reduced interaction with LexA-Gpa1 (Figure 1A). Neither mutant allowed mating of SDY109 at 30° (Figure 1B), nor did they induce the "shmoo" morphology characteristic of mating-competent cells (data not shown).

TABLE 2

Interaction defects of Ste4p mutants

	GAD	Ste4	D224A	L49P	L65S	D62N
LexA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.37 ± 0.0050	0.98 ± 0.52
Gpa1	0.48 ± 0.23	260 ± 0.85	4.7 ± 1.4	330 ± 46	240 ± 21	130 ± 33
Ste18	1.4 ± 1.4	400 ± 93	370 ± 7.1	84 ± 20	110 ± 4.6	280 ± 19
Ste5	1.9 ± 0.20	350 ± 43	150 ± 18	1.1 ± 0.51	2.8 ± 0.12	160 ± 31
Akr1	0.0 ± 0.0	94 ± 1.4	84 ± 3.0	0.14 ± 0.14	44 ± 10	160 ± 5.0

Quantitative β-galactosidase assays were carried out for two-hybrid interactions of GAD-Ste4 or GAD-Ste4 mutants in combination with the LexA fusions as indicated, in strain M364-2C. Numbers given are the average of three samples in arbitrary units.

Sequencing revealed that mutant 39 had two amino acid changes, L49P and D224A, whereas mutant 57 had a single amino acid change, L65S. L49P, L65S, D224A and the previously identified dominant-negative mutation D62N (Leberer *et al.* 1992b) were recreated singly into wild-type Ste4p fused to GAD. Quantitative β-galactosidase assays in yeast strain M364-2C confirmed that L49P and L65S were responsible for the defect in Ste5p interactions, while D224A was responsible for the interaction defect of mutant 39 with LexA-Gpa1 (Figure 1A; Table 2). D62N, however, showed no major defect in any interaction. The L49P mutation may cause a significant structural change; therefore a relatively conservative change of L49 to alanine was constructed. Ste4 L49A was similar to L49P in its defect with LexA-Ste5 (Figure 1C), suggesting that the role of L49 in the interaction with Ste5p is significant.

As an additional control, the mutant GAD-Ste4 constructs were tested in their interactions with LexA-Akr1. In quantitative assays, Ste4 L65S and D62N maintained strong interactions with Akr1p (Table 2). However, L49P and L49A both had a significantly reduced interaction with LexA-Akr1 (Figure 1C and Table 2). Therefore, although L49 and L65 mutations are similarly defective in their interactions with Ste5p, they are distinguishable in their interactions with Akr1p.

Mapping of the Ste4p mutations on a structural model: Comparison with the crystal structure of the bovine Giα1β1γ2 heterotrimer (Wall *et al.* 1995) suggests both L49 and L65 may contribute to hydrophobic coiled-coil interactions with Ste18p (Figure 2). Consistent with this role, Ste4 L49P, L49A, and L65S had reduced affinities for Ste18p in the two-hybrid assays (Table 2 and Figure 1C), confirming the importance of these leucines in the hydrophobic interface of the coiled-coil. However, the effect of these mutations on Ste18p binding was minor compared to the effect on Ste5p binding. The D62 and K55 residues, required for Ste20p binding, are also in this region of Ste4p but lie on the opposite face of the Ste4p helix with their side chains pointing away from the Ste18p subunit. D224 could potentially form a hydrogen bond with Gpa1p (Figure 2; Sondek *et al.* 1996). The reduced interaction between Ste4 D224A and LexA-Gpa1 is consistent with loss of a hydrogen bond at D224.

Ste4 L65S is temperature-sensitive: Strains expressing the Ste4 L65S mutant as the only Ste4p species were unable to mate at 30°, but mated efficiently when incubated at 20° (see below). In two-hybrid assays, quantitative β-galactosidase measurements showed that while at 30° the interaction between Ste4 L65S and the LexA-Ste5 construct was barely measurable, at 20° this interaction was restored almost to the level seen with wild-type GAD-Ste4 (Table 3). The interactions of GAD-Ste4, Ste4 L49P, and Ste4 L65S with LexA-Ste18 were all slightly higher at 20° than at 30°. However, the ratios of activities of the Ste4p mutants compared to the GAD-Ste4 control were not significantly different at the two temperatures. The interactions between Ste4p and Ste4 L65S with Akr1p were both slightly increased at the higher temperature. Therefore, the temperature-sensitive signaling phenotype of Ste4 L65S correlates directly with a tightly temperature-sensitive two-hybrid interaction of this molecule with Ste5p, and suggests that the mating defect of this Ste4p mutant at 30° is not due to a reduced interaction with Ste18p.

Ste5p residues defective in the interaction with Ste4p: Random mutagenesis of Ste5p failed to identify mutants able to suppress the Ste4 L49P or Ste4 L65S mutations. Therefore we investigated the effect of directed mutations in Ste5p on the interaction with Ste4p. A schematic representation of Ste5p is shown in Figure 3A. The first 214 amino acids of Ste5p are sufficient for interaction with Ste4p in the two-hybrid assay (Whiteway *et al.* 1995). Of particular interest was the RING-H2 domain (Figure 3, A and B), a motif implicated in protein-protein interactions. Although residues 1–214 do not include the entire RING-H2 homology region, mutation C177S in the LexA-Ste5 construct abolished the interaction with GAD-Ste4, implicating the RING-H2 structure in the Ste4p-Ste5p interaction (Table 4). However, this mutation may cause a major local change in the structure of Ste5p, and therefore we investigated the effect of more subtle mutations in this region. The dramatic effect of the relatively conservative Ste4p L65S and L49A mutations raised the possibility that hydrophobic resi-

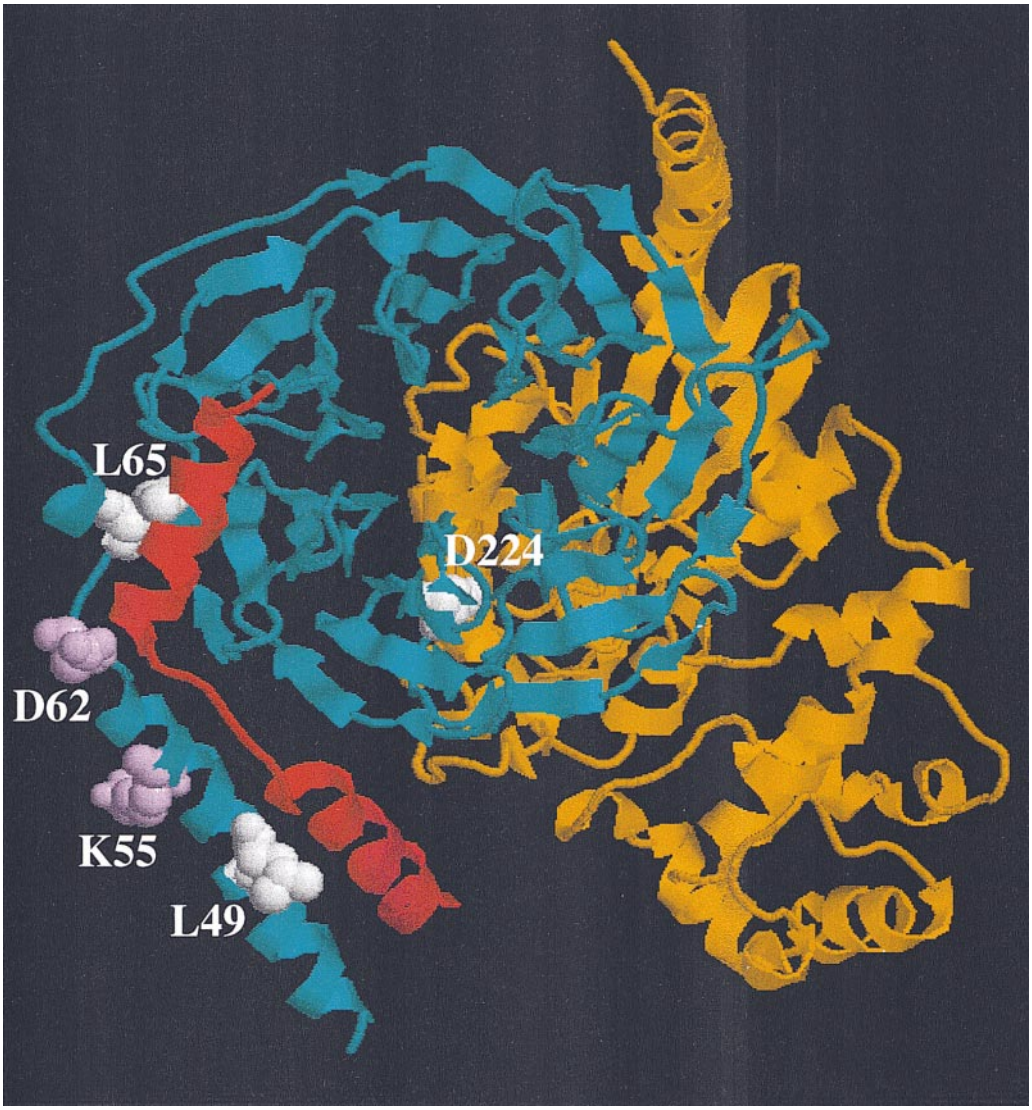

Figure 2.—Model of the Gpa1/Ste4/Ste18 complex, based on the crystal structure of the Gαi1β1γ2 heterotrimer, showing Gpa1p in orange, Ste4p in blue, and Ste18p in red. Residues L49, L65, and D224 are indicated in white. K55 and D62, required for the interaction with Ste20p, are indicated in violet.

dues may participate in the Ste4p-Ste5p interface. Many hydrophobic interactions, such as those mediated by leucine-zipper coiled-coils, are highly sensitive to mutation of leucine residues. Only one short region in the amino terminus of Ste5p (residues 125–141) has some of the qualities of an amphipathic helix. Within this region, mutation of L128A and L136A had no measurable effect on Ste4p binding (data not shown).

In contrast, conservative mutations in the RING-H2 domain, depicted in Figure 3B, affected Ste4p binding

TABLE 3

The Ste4 L65S-Ste5 interaction is temperature sensitive

	GAD	Ste4	L49P	L65S
Ste18 20°	5.8 ± 1.0	590 ± 20	390 ± 9.9	480 ± 53
Ste18 30°	0.0 ± 0.0	450 ± 2.2	150 ± 10	230 ± 41
Akr1 20°	0.0 ± 0.0	170 ± 8.7	0.0 ± 0.0	200 ± 3.6
Akr1 30°	0.90 ± 0.25	280 ± 37	4.5 ± 0.0	360 ± 100
Ste5 20°	0.0 ± 0.0	200 ± 10	0.0 ± 0.0	180 ± 45
Ste5 30°	0.10 ± 0.20	250 ± 57	1.7 ± 0.31	3.5 ± 2.2

Quantitative β-galactosidase assays for two-hybrid interactions of GAD-Ste4 or GAD-Ste4 L49P and L65S mutants with LexA-Ste18, lexA-Akr1, and LexA-Ste5. The transformants (of strain M364-2C) were grown with shaking in liquid culture for 48 hr, at the temperatures indicated, then assayed for β-galactosidase activity. Numbers given are the average of three samples in arbitrary units.

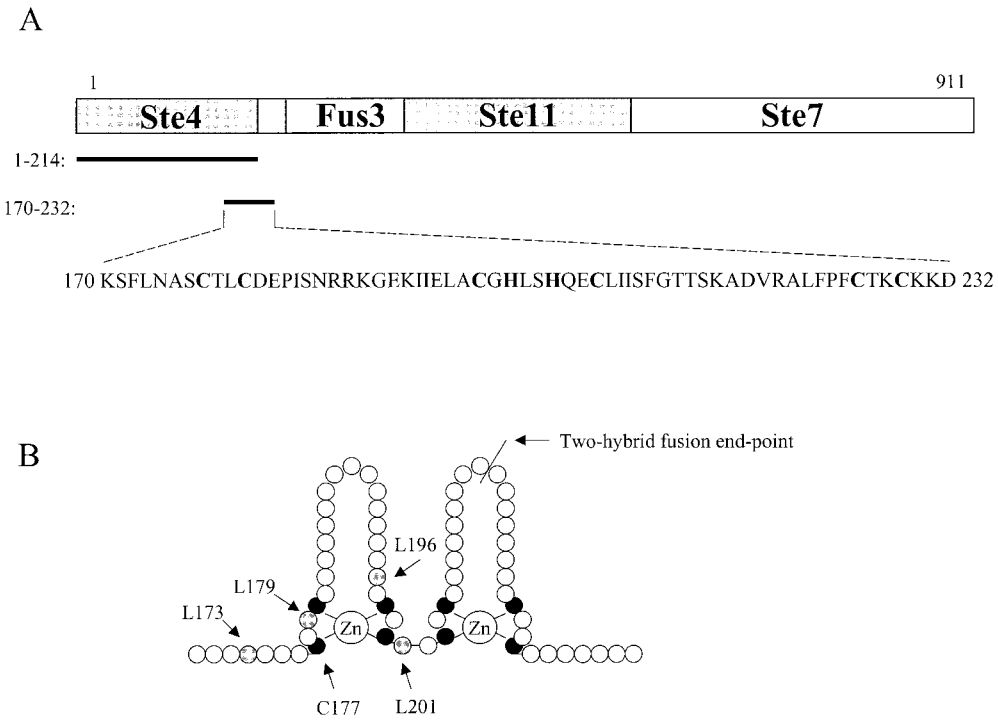

Figure 3.—(A) Schematic representation of Ste5p showing regions implicated in interactions with Ste4p, Fus3p, Ste11p, and Ste7p. Black bars show the region of Ste5p included in the LexA fusion (1–214) and the region of the RING-H2 domain (170–232). Cysteines and histidines of the RING-H2 consensus are depicted in bold type. (B) Cartoon showing possible arrangement of the RING-H2 domain and location of mutations made in this study in gray. The cysteines and histidines of the RING-H2 consensus are in black.

(Figure 4). Ste5 L196A showed a small but measurable defect in the interaction with wild-type Ste4p, and with Ste4 D62N, as observed by a slightly reduced size in the discs of growth on 3-amino-triazole plates. However, when combined with the Ste5 L196A mutant, the Ste4 L65S interaction observed at 20° was abolished. Ste5 L201A had a less severe but measurable defect in the interaction with Ste4 L65S at 20°. A Ste5 L196A/L201 double mutation was similar to the single L196A mutation. Mutation of L173A or L179A had no measurable effects (data not shown).

A synthetic mating defect of Ste4p and Ste5p mutants: To ascertain the biological significance of the Ste4p and Ste5p two-hybrid mutants, Ste4 L49P, L65S, D62N, and L49A were reconstructed in Ste4p tagged with the HA epitope; Ste5 L196A, L201A, and the L196A/L201A double mutant were reconstructed in full-length Ste5p. The constructs were expressed in a strain (SDY110) deleted for chromosomal *STE4* and *STE5* and signal transduction was assessed in mating assays. Strains expressing wild-type Ste4p, in combination with wild-type Ste5p, or any of the Ste5p mutants, could mate at all temperatures tested (Figure 5A). However, strains expressing Ste4 L49P as the only Ste4p species failed to mate at any temperature, irrespective of the Ste5p species. Strains expressing Ste4 L65S in combination with wild-type Ste5p failed to mate at 30°, but could mate at lower temperatures. However, when the Ste5 L196A mutant was introduced, mating was not observed at any temperature.

The Ste4 D62N mutant was also found to be temperature-sensitive, supporting weak mating in combination with wild-type Ste5p at lower temperatures. This signaling was also abolished in combination with the Ste5 L196A mutant. Because D62N alone has no apparent defect in the interaction with wild-type Ste5p, this synthetic effect may be due to combining the enfeebled signaling capability of D62N with the slight interaction defect of Ste5 L196A.

Immunoblotting confirmed that the Ste4p and Ste5p mutant proteins were all expressed at levels similar to the wild-type proteins (Figure 5, B and C). Therefore the signaling defects are unlikely to be caused by instability or reduced expression of the Ste4p and Ste5p mutant proteins.

TABLE 4

A Ste5p RING-H2 domain mutation abolishes the interaction with Ste4p

	GAD	Ste4
Ste5	2.0 ± 0.30	120 ± 7.1
Ste5 C177S	0.30 ± 0.070	0.40 ± 0.020

Quantitative β-galactosidase assays were carried out in strain M364-2C for the interaction of GAD-Ste4 with either LexA-Ste5 or LexA-Ste5 C177S. Numbers are the average of two samples, in arbitrary units.

DISCUSSION

We have identified two leucine residues in the amino terminus of Ste4p that appear to be specifically required for the interaction with Ste5p. Both residues contribute

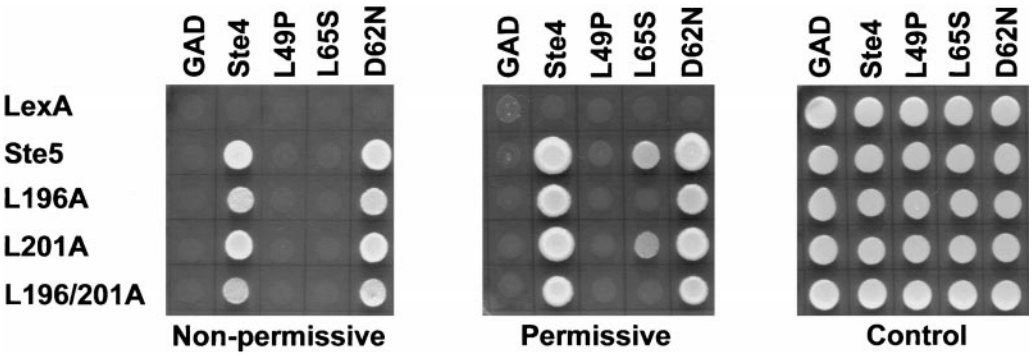

Figure 4.—Growth assay for two-hybrid interactions of GAD-Ste4 and LexA-Ste5 proteins and their mutant derivatives as described in materials and methods. Test plates were incubated at either 20° (permissive) or 30° (nonpermissive). The control plate contained histidine and no 3-amino-triazole and was incubated at 30°.

to the hydrophobic coiled-coil interface that Ste4p is predicted to form with Ste18p on the basis of homology and modeling of the Gpa1p/Ste4p/Ste18p complex on the crystal structure of mammalian Giα1β1γ2 (Wall *et al.* 1995). Identification of this region of Ste4p in the screen may be predictable: previously identified dominant negative mutations have implicated the coiled-coil in pheromone signaling (Leberer *et al.* 1992b; Whiteway *et al.* 1992; Grishin *et al.* 1994); furthermore, the Ste4p association with Ste5p requires Ste18p (Whiteway *et al.* 1995), whereas the Ste4p association with Gpa1p does not (Clark *et al.* 1993). However, the precise nature of the Ste5p-defective residues is surprising, because in the free Gβγ structure these leucines are not obviously accessible for direct interaction with other proteins. Nevertheless, several pieces of evidence suggest that these findings are significant. First, in the quantitative two-hybrid interactions, L49P and L65S were almost completely defective in the Ste5p interaction, while having no detectable interaction defect with Gpa1p and a relatively slight interaction defect with Ste18p. Also, the temperature-sensitive mating defect caused by the Ste4 L65S mutant correlates with a striking temperature-sensitive interaction defect with Ste5p, whereas no significant temperature-sensitive two-hybrid interactions were observed with the other interacting partners. Finally, the mutations that we engineered in Ste5p provide supportive evidence that a hydrophobic interface may be involved in the interaction between the two proteins. The combination of the subtle Ste4 L65S and Ste5 L196A mutations has an effect both on the two-hybrid interaction and on signaling at the "permissive" temperature of the L65S mutant that is greatly in excess of the sum of the two individual mutations.

Since Ste18p is required for the interaction between Ste4p and Ste5p, it cannot be ruled out that a small defect in the Ste4p-Ste18p interaction in the two-hybrid assay reflects a conformational change sufficient to displace Ste5p binding from a distinct site on the Ste4 molecule. L49A and L65S are predicted to be structur-

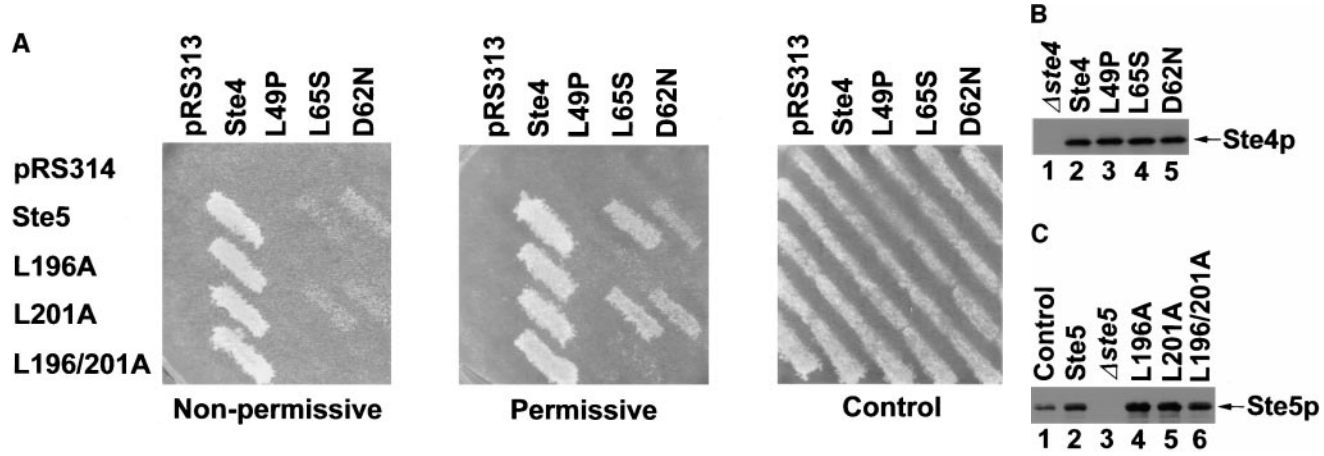

Figure 5.—Synthetic mating defects of Ste4p and Ste5p mutants. (A) Mating assays of SDY110 cells expressing Ste4p and Ste5p wild-type or mutant proteins in the combinations shown. The control panel shows the haploid SDY110 transformants. The mating reactions were incubated either at 27.5° (permissive) or 30° (nonpermissive) for 16 hr, before replica-plating onto diploid-selective plates. (B) Quantification of HA-Ste4 and mutant HA-Ste4 protein levels. Samples are extracts of SDY109 transformed with the following constructs: pRS313 (lane 1), pL55 (lane 2), pL55 L49P (lane 3), pL55 L65S (lane 4), and pL55 D62N (lane 5). The blot was probed with 16B12 monoclonal antibody. (C) Quantification of Ste5p and mutant Ste5p protein levels. Lane 1 is an extract of W303-1B. All other samples are extracts of SDY110 transformed with the following constructs: pRS314-STE5 (lane 2), pRS314 (lane 3), pRS314-STE5 L196A (lane 4), pRS314-STE5 L201A (lane 5), and pRS314-STE5 L196A/L201A (lane 6). The blot was probed with anti-GST-Ste5 polyclonal antibody.

ally conservative but still affect the interaction with Ste18p, making it difficult to distinguish a direct from an indirect effect on Ste5p binding. However, it seems unlikely that such a dramatic effect on Ste5p binding would be caused by a relatively minor defect in Ste4p-Ste18p affinity. Furthermore, in our screen we identified other Ste4p mutants that were more severely defective in the Ste18p interaction but still able to bind Ste5p and signal (data not shown). Also, the Ste4p-Akr1p interaction was inhibited by the L49 mutations, consistent with the observation that this interaction requires Ste18p (Pryciak and Hartwell 1996) and may involve the Gβγ coiled-coil. However, L65S did not affect Akr1p binding; therefore, the Ste4p-Ste5p and Ste4p-Akr1p interactions are distinguishable, and the Ste5p interaction defect caused by L65S is unlikely to be due to a major structural perturbation of the coiled-coil. Taken together these arguments may suggest a direct involvement of Ste4p L49 and L65 in the Ste5p interaction.

L49 and L65 are in close proximity to, but distinct from, residues identified as dominant negative mutations in Ste4p (K55, K59, and D62; Leberer *et al.* 1992b) and Ste18p (R34, E37, R43, A56, R48; Whiteway *et al.* 1992; Grishin *et al.* 1994), which are mostly charged and lie on the outer, solvent-exposed faces of the Gβ and Gγ helices in the coiled-coil. The D62N and K55E mutations are defective in binding Ste20p (Leeuw *et al.* 1998). However, D62N has no defect in binding Ste5p in the two-hybrid assay (Whiteway *et al.* 1995 and this work). Therefore the L65 and L49 mutants appear to define a new signaling interface of Ste4p. The mutations are sufficiently far apart structurally to suggest an extended interface that may require unwinding or deformation of the Ste4p/Ste18p coiled-coil. The relatively high thermal parameters of the coiled-coil render this possibility feasible (Sondek *et al.* 1996). An interesting implication is that the crystal structures of Gβγ complexes may not reflect the actual conformation Ste4p/Ste18p adopts in the cell, if Ste4p is in fact constitutively associated with Ste5p.

The close proximity of the Ste20p and Ste5p binding sites is also interesting, and indicates that Ste4p may recruit Ste20p precisely to the vicinity of Ste5p and proteins known to be bound to Ste5p, such as Ste11p. The results may have implications for mammalian Gβγ signaling. Although no studies have yet revealed direct involvement of mammalian Gβγ coiled-coils in effector binding, the evolutionary conservation from yeast to mammals, together with recent identification that the Ste4p-binding site of Ste20p is conserved in mammalian Gβ subunits (Leeuw *et al.* 1998), implies that functional mechanisms are also conserved. The coiled-coil is the region of highest variability between Gβ and between Gγ subtypes enabling, by combinatorial dimerization, many possible effector-binding specificities. The leucines that define the hydrophobic interface are among the most invariant residues in the coiled-coil. Therefore, although we identified a role for these leucines in Ste5p binding, other residues may be involved in mediating specificity in Gβγ-effector interactions.

We have shown that residues in the RING-H2 domain of Ste5p are likely to contact Ste4p, although only part of the RING-H2 consensus is present in the LexA-Ste5 construct. If this model is correct, only the first of the two RING-H2 fingers is required to bind Ste4p. Two recent articles have also implicated the Ste5p RING-H2 domain in binding Ste4p. Ste5 C177S or Ste5 C177A C180A mutants were unable to complement the mating defect of a *ste5*Δ strain (Inouye *et al.* 1997), and a Ste5 C180A mutant failed to bind Ste4p but could bind Ste11p, Ste7p, and Fus3p (Feng *et al.* 1998). These articles conflict over the role of Ste4p in dimerization of Ste5p. The C177A C180A mutant fails to self-associate, but when dimerization is enforced by fusion to GST, signaling can occur in the absence of pheromones, leading Inouye *et al.* (1997) to propose that Ste4p binding is required for Ste5p dimerization. However, the Ste5 C180A mutant fails to bind Ste4p but can self-associate, suggesting that Ste4p binding is not essential for Ste5p dimerization. We also showed that the Ste5 C177S mutant failed to bind Ste4p, in agreement with both these reports. However, the L196A and L201A mutations reported here are less likely to perturb the Ste5p structure, and provide stronger evidence that the RING-H2 structure contacts Ste4p directly. The fact that these Ste5p mutants do not appear to confer a signaling defect in the presence of wild-type Ste4p suggests that they are competent to dimerize, in agreement with evidence that the region of Ste5p required for homodimerization is separable from the Ste4p-binding site (Feng *et al.* 1998).

The RING-H2 domain of Ste5p does not contain the consensus motif Gln-X-X-Glu-Arg identified as a putative Gβγ-binding site in various mammalian proteins, including adenylyl cyclases, β-adrenergic receptor kinases, phospholipase Cβ, and a variety of ion channels (Chen *et al.* 1995; Ma *et al.* 1997, and references therein). Similarly, RING-H2 finger motifs have not been identified in these Gβγ targets, and indeed, structural homologues of Ste5p remain to be identified. Gβγ subunits are likely to have several distinct interaction interfaces as suggested by the number of Ste4p-binding partners and the diverse mammalian Gβγ targets. Identification of the coiled-coil in effector binding is novel. However, interactions between zinc fingers and leucine zippers have been reported for a number of transcription factors, such as the glucocorticoid receptor and c-Jun (reviewed in Schule and Evans 1991) and the heterologous proteins GAL4 and c-Jun (Sollerbrant *et al.* 1995), showing that these structures can heterodimerize. Although the Ste4p- and Ste5p-interacting regions are not directly homologous to the leucine zippers and zinc fingers of these transcription factors, the Ste4p-Ste5p interaction may represent a more general protein-protein interaction mechanism.

We thank Cunle Wu and Ekkehard Leberer for plasmids, access to unpublished data, and useful discussions, and Robert Larocque for supplying plasmids. We also thank John Hillman for photography and Alan Lewis for assistance with the RasMol program. We are grateful to Mike Romanos and Mark Payton for support of this work.

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Communicating editor: F. Winston