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The Leu-132 of the Ste4(Gβ) subunit is essential for proper coupling of the G protein with the Ste2 α factor receptor during the mating pheromone response in yeast

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Abstract In order to identify amino acid residues of Ste4p involved in receptor recognition and/or receptor-G protein coupling, we employed random in vitro mutagenesis and a genetic screening to isolate mutant Ste4p subunits with altered pheromone response. We generated a plasmid library containing randomly mutagenized *Ste4* ORFs, followed by phenotypic selection of ste4p mutants by altered α pheromone response in yeast cells. Subsequently, we analyzed mutant *ste4-10* which has a replacement of the almost universally conserved leucine 132 by phenylalanine. This residue lies in the first blade of the β propeller structure proposed by crystallographic analysis. By overexpression experiments we found that mutant ste4p subunit triggers the mating pathway at wild type levels in both wild type and receptorless strains. When expressed in a *ste4* background, however, the mutant G protein is activated inefficiently by mating pheromone in both a and α cells. The mutant ste4-10p was tested in the two-hybrid system and found to be defective in its interaction with the Gpa1p, but has a normal association with the C-termini end of the Ste2p receptor. These observations strongly suggest that the Leu-132 of the Ste4p subunit is essential for efficient activation of the G protein by the pheromone-stimulated receptor and that this domain could be an important point for physical interaction between the Gβ and the Gα subunits.

Key words: Ste4; Ste2; G protein; Signal transduction; Structure-function; Yeast; *Saccharomyces cerevisiae*

1. Introduction

Signal transduction between seven transmembrane segment receptors and a variety of intracellular effectors, is mediated by heterotrimeric G proteins composed of α, β and γ subunits. Binding of ligand causes a conformational change in the receptor that activates the G protein rendering a GTP-activated Gα subunit dissociated from the Gβγ complex. Both, the GTP-Gα and the Gβγ moieties activate intracellular effectors. Hydrolysis of GTP by intrinsic activity of Gα subunit induces reassembling of subunits and the G protein becomes inactive [1,2].

Crystal structures of $G\alpha_{i1}\beta_1\gamma_2$ [3], $G\alpha_t\beta_1\gamma_1$ [4] and free $G\beta_1\gamma_1$ [5] reveal the molecular folding of G protein subunits and show that the conformations of Gα alone [6] and in the trimeric complex are different. They also reveal that the N-terminal region and catalytic domain of the Gα make extensive contacts with Gβ. The Gβ subunit folds in a highly symmetric β propeller containing seven β sheets that form the blades of the propeller. Each blade consists of four antiparallel strands radiating outwards from a central core [5]. Crystal structures also reveal that the N-terminal region of Gβ subunit is involved in the interhelical dimerization with the N-terminal of Gγ subunit by forming an α helical coiled-coil structure as it was predicted [7,8].

Besides contact surfaces between the G protein subunits revealed by crystal structures there are also biochemical and genetic evidences that show direct interactions of receptor with both Gα and Gβγ [9–12] that support the general accepted mechanism of G protein activation by receptor occupancy, in which has been described that Gα has a central and essential role (reviewed [13,14]). Although there is general agreement on the events of receptor/G protein transduction pathway, a number of questions still need to be answered, particularly regarding the influence of Gβγ in the receptor-stimulated Gα activation-inactivation cycle.

The *Ste4* gene encodes the Gβ subunit [15] that associates with the *Gpa1* or *Scg1* (Gα) [16,17] and *Ste18* (Gγ) [15] gene products, forming the heterotrimeric G protein involved in the pheromone response pathway in the yeast *Saccharomyces cerevisiae*. This G protein is the same in **a** and α cells. *Mat***a** cells respond to α pheromone which triggers the pathway by interacting with the α pheromone receptor Ste2p, and *Mat*α cells respond to **a** pheromone that interacts with the **a** pheromone receptor Ste3p. *Ste2* and *Ste3* encode membrane proteins that belong to the superfamily of seven-transmembrane-spanning G protein coupled receptors [18,19]. Mating response is mediated by free Ste4p/Ste18p dimer, which can be generated by pheromone induction, Gpa1p inactivation or *Ste4* overexpression [20,21]. These events lead to transcriptional activation of genes, changes in morphology (*shmoo* formation), and growth arrest. Gpa1p plays a negative role in the pathway and *gpa1* cells exhibit constitutive growth arrest and are able to mate [22–24].

Taking advantage of the genetically tractable model of the yeast pheromone response pathway, here we described the isolation and characterization of a *Ste4* mutant with an altered pheromone response and we describe genetic evidence that shows that Ste4p has an important role in the proper

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functional coupling between the G protein and the pheromone-stimulated receptor.

2. Materials and methods

2.1. Strains and media

The following *S. cerevisiae* strains were provided by J. Kurjan: W303-1A (*Mat***a**, *ade2, his3, leu2, trp1, ura3, can1-100*); W303-1B (*Mat*α, *ade2, his3, leu2, trp1, ura3, can1-100*); W303-3A (*Mat***a**, *ade2, his3, ura3, trp1, can1-100, ste2::Leu2*) and 70 (*Mat*α, *thr3*). Strains W303-5A (*Mat***a**, *ade2, his3, leu2, trp1, can1-100, ste4::Ura3*) and W303-6B (*Mat*α, *ade2, his3, leu2, trp1, can1-100, ste4::Ura3*) were constructed by gene disruption of the *Ste4* locus in the W303-1A and W303-1B strains, respectively. EGY48 (*Mat*α, *his3, trp1, ura3-52, leu2::pLeu-LexAop6*) was used as a recipient for two-hybrid system plasmids. *Escherichia coli* strain DHα5 was used to propagate ampicillin-resistant plasmids.

YPD and YPGal media consisted of 1% yeast extract, 2% bactopeptone and 2% glucose or 2% galactose, respectively. SD minimal medium consisted of 0.65% yeast nitrogen base without amino acids (DIFCO) and 2% glucose. SGal medium was the same except for the substitution of 2% galactose for glucose. SD and SGal media were supplemented with the required nitrogen bases and amino acids (50 μg/ml) to select for yeast cells carrying the desired plasmids.

2.2. Plasmids

Plasmids pJG4-5, pEG202 and pSH18-34 [25] were used for the two-hybrid interaction experiments. pGALHis and pGALHis*Ste4* were previously described [26]. pGALUraGpa1 was constructed by subcloning a *Bam*HI-*Nco*I *Gpa1* fragment into the pGALUra vector [26], digested with the same enzymes. pCUPGpa1 was constructed by digesting pGALUraGpa1 with *Bam*HI, filled in with Klenow and digested with *Nco*I. The fragment carrying the *Gpa1*ORF was then subcloned into the pCUP vector previously digested with *Asp*718, Klenow filled, and digested with *Nco*I.

2.3. Fus1-LacZ fusion

A 0.6-kb fragment carrying the *Fus1* promoter and the first 324 bases of the ORF encoding 108 amino acids of the N-terminus of the protein was amplified by PCR. The PCR product was then treated with Klenow enzyme, phosphorylated at its 5′ termini and digested with *Pst*I (a naturally occurring restriction site at position −165). The product was then subcloned into YEp351 [27], prepared by digesting with *Sal*I, filled in with Klenow and digested with *Pst*I to give rise to YEp351-Fus1. *LacZ* was obtained by PCR mediated amplification using oligodeoxynucleotides designed to amplified a 3072-bp product from *E. coli* chromosomal DNA strain HB101. The PCR product was treated with Klenow enzyme, digested with *Xba*I and ligated into the YEp351-Fus1 plasmid previously digested with *Sma*I and *Xba*I. This rendered an in frame fusion product containing 108 N-terminal amino acids of Fus1p and 1016 amino acids of the β-galactosidase under the control of the *Fus1* promoter.

2.4. PCR-mediated random mutagenesis

The *Ste4* open reading frame was subjected to random mutagenesis by PCR-amplification using in the reaction 2mM Mn^{2+} and 250 μM dNTPs except for dATP which was 125 μM. *Ste4* was amplified by using oligodeoxynucleotides directed to 5′ and 3′ ends that introduced at the same time *Eco*RI (position −7) and *Bam*HI (position 1282) restriction sites, respectively. 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 *Eco*RI and *Bam*HI and subcloned into the pGALHis vector previously digested with the same enzymes. This placed the *Ste4* PCR products under the control of the *Gal1* promoter (Fig. 1).

2.5. Pheromone response assays

Response to α factor and **a** factor was tested by monitoring cell number and percentage of unbudded cells. Strains to be tested were grown until midlog phase in selective medium (SD plus the required amino acids). An aliquot of these cultures was transferred into SGal (plus required amino acids) and incubated at 30°C for 1 h to induce expression of *Ste4*. 1×10^6 cells were transferred to 1 ml of fresh medium (SGal) containing various concentrations of α or **a** pheromone. Cell number and percentage of unbudded cells were determined at different times using a cell counting chamber after brief sonication. α Pheromone was obtained from Sigma, and **a** pheromone was obtained as the supernatant of a saturated culture of **a** cells (strain W303-1A). One unit of **a** pheromone was referred as the volume of medium that produces a growth inhibition halo of 1 cm of diameter on a lawn of 1×10^4 α cells (strain 70).

2.6. Interaction assays

Assays of physical interaction were done with the LexA-B42 two-hybrid system as described [25]. The full length ORFs of *Ste4* and *ste4-10* were subcloned into pJG4-5 as in-frame *Eco*RI-*Xho*I fragments (positions −5 and 1284, respectively) obtained by PCR mediated amplification. PCR products of *Gpa1* and *Ste2* were subcloned into pEG202. *Gpa1* was obtained as an *Eco*RI-*Xho*I fragment (positions −2 and 1428, respectively), and *Ste2* was obtained as a *Bam*HI-*Xho*I fragment (positions −1 and 1311, respectively). A 269-bp *Alu*I fragment (from position 977 to 1246) encoding a part of the C-terminus end of the Ste2p receptor was subcloned into pEG202, previously digested with *Eco*RI and treated with Klenow enzyme. The *Eco*RI-*Xho*I PCR product of *Gpa1* was also subcloned into pJG4-5 digested with the same enzymes. The *S. cerevisiae* endochitinase gene (*Cts1-2*), amplified by PCR using oligodeoxynucleotides that introduce *Eco*RI (position −2) and *Xho*I (position 1994), was subcloned into pEG202 to use it as interaction negative control. EGY48 strain was transfected with two-hybrid plasmids and grown in SGal medium at 30°C for 12 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.

2.7. Other

Transformation of yeast was done by the alkali cation method [28]. Recombinant DNA techniques were essentially as described [29]. Automated sequencing was done by the ABI-PRISM 310 (Perking-Elmer). β-Galactosidase activity was determined as described [30].

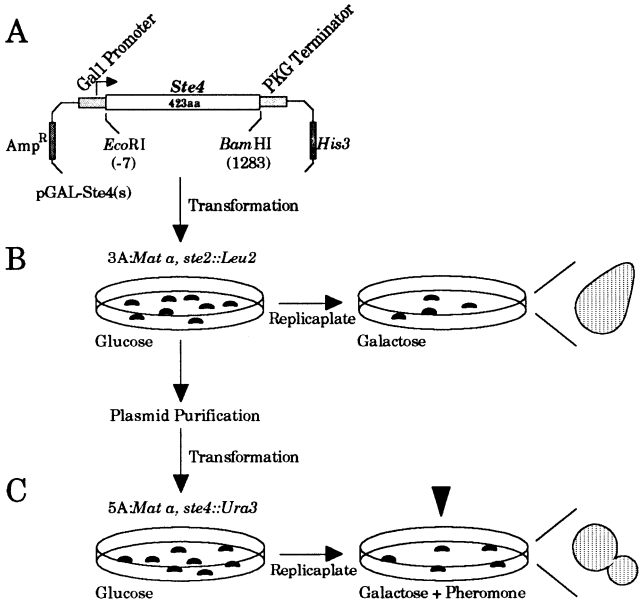

Fig. 1. Strategy for selection of *ste4* mutants with deficient α pheromone response. A: pGALHis plasmid containing the PCR-mutagenized *ste4* ORFs, under the control of the *Gal1* promoter. B: *ste2* Strain was transfected with pGALHis-based library, plated on SD medium plus the required amino acids and incubated at 30°C for 48 h. Colonies were replicaplated to SGal medium and incubated in the same conditions. Plasmids from growth-arrested clones (shmoo morphology) were purified, mixed and amplified in *E. coli* DHα5 strain. C: *ste4* Cells were transfected with a mixture of purified plasmids and plated on SD plus required amino acids. After 48 h incubation at 30°C colonies were replicaplated to SGal containing 10 ng/ml α pheromone and incubated 24 h in the same conditions.

3. Results

The *Ste4* ORF was mutagenized by amplification using the polymerase chain reaction under conditions that reduce the fidelity of DNA synthesis by *Taq* DNA polymerase (see Section 2). By using oligodeoxynucleotides that introduce *Eco*RI and *Bam*HI restriction sites at positions −7 and +1282, respectively, we were able to generate a random mutant library of 400 independent clones into the pGALHis vector, placing *Ste4* products under the control of the galactose inducible promoter (Fig. 1A). Fidelity of amplification by *Taq* polymerase in normal conditions has been calculated to be 8.0×10^{-6} mutation frequency/bp [31]. Using the amplification conditions here described we obtained a frequency of point mutations of 0.2% based on DNA sequence analysis of a small collection of randomly picked clones. Transitions were estimated up to 70% of the missense mutations while no deletions or insertions were detected.

We then devised a strategy for genetic selection of *Ste4* mutants with altered α pheromone response (Fig. 1). Screening of the library was done first by transfecting a *ste2* mutant followed by replica-plating to galactose medium to induce expression of *Ste4* PCR products. In this first step we looked for clones able to induce growth arrest and *shmoo* morphology by overexpression (Fig. 1B). This screening allowed us to select clones with an apparent normal ability to activate the pathway downstream the G protein. A collection of the plasmids from the selected clones was used to transfect a *ste4* mutant (Fig. 1C). After replica-plating to galactose medium containing α pheromone we select for budding clones showing normal ability to form colonies. By its limited response to α pheromone, we selected mutant *ste4-10* for further and detailed characterization.

Overproduction of Ste4p in wild type and *ste2* cells induces transcription of genes, one of which is *Fus1*, growth arrest and *shmoo* morphology [15,16]. We explored whether the ste4-10 mutant subunit was able to induce these responses at the same level as wild type subunit does. Fig. 2 shows growth kinetics on SGal medium of *ste2* cells carrying pGALHis constructs. In these conditions both Ste4p and ste4-10p were able to arrest the growth of the cells and induce *shmoo* morphology. Induction of the fusion *Fus1-LacZ* was indistinguishable in cells carrying both alleles (not shown). These results indicate that the mutant ste4-10 subunit had preserved its normal capacity to bind Ste18p and to signal the downstream effector.

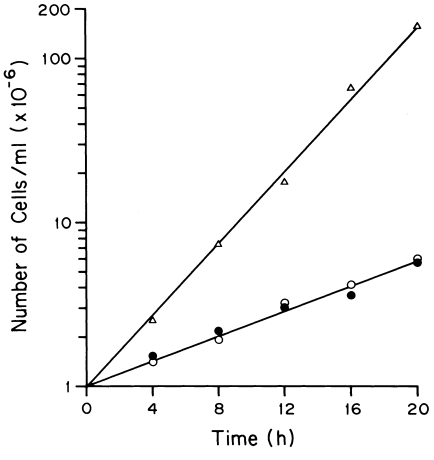

Fig. 2. Effect of overexpression of *Ste4* and *ste4-10* on growth properties of *ste2* cells in liquid cultures. Cells carrying pGALHis constructs were grown until midlog phase in SD plus the required amino acids. An aliquot of this culture was transferred for 1 h to SGal medium to induce pGALHis constructs. 1×10^6 Cells were transferred to 5 ml of fresh SGal medium. Cell number was determined at the indicated times using a cell counting chamber. (△) pGALHis; (●) pGALHis-Ste4; (○) pGALHis-ste4-10.

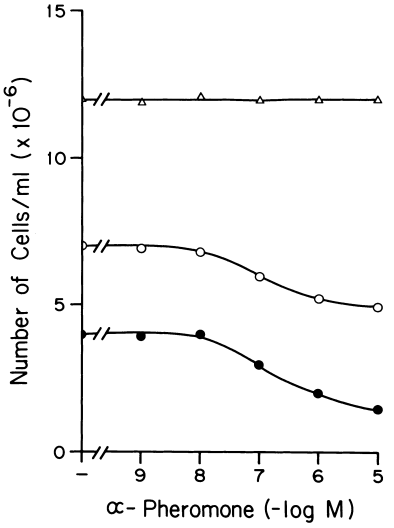

Fig. 3. Response to α pheromone of *ste4* cells carrying pGALHis constructs. Cells were grown until midlog phase in SD plus the required amino acids. An aliquot of the culture was transferred to SGal medium to induce pGALHis constructs. 1×10^6 Cells were transferred to 1 ml of SGal medium containing different concentrations of α pheromone and incubated for 6 h at 30°C with shaking. Cell number was determined using a cell counting chamber. (△) pGALHis; (●) pGALHis-Ste4; (○) pGALHis-ste4-10.

Addition of α pheromone to *Mat* **a**, *ste4* cells has no effect on their growth rate [15]. Expression of wild type *Ste4* led after a time of about 4 h to growth arrest. This effect was accelerated in a dose-dependent manner by α pheromone (Fig. 3). Cells expressing, under identical conditions the *ste4-10* allele showed a right shift in the dose-response curve to α pheromone (Fig. 3). This was measured by both growth arrest (cell number, Fig. 3) and *Fus1* induction (Fig. 4). The same is true for α cells carrying the mutant allele responding

Table 1
a Pheromone-dependent *Fus1* induction on α cells[a] carrying *Ste4* constructs

a Pheromone (U)[b]	β-Galactosidase activity (U/mg protein)[c]	
	Plasmids	
	Ste4	Ste4-10
0	45 ± 5	40 ± 7
1	140 ± 16	80 ± 12
10	175 ± 13	105 ± 10

[a]Strain W303-6B (*Mat*α), was grown until midlog phase in SD medium. An aliquot of this culture was transferred to SGal for 1 h to induce expression of pGALHis-*Ste4* constructs. 1×10^7 Cells were then transferred to 2 ml of fresh SGal medium containing **a** pheromone and incubated for 6 h at 30°C.
[b]1 Unit of **a** pheromone corresponds to the volume of a saturated **a** cells culture that produces a growth inhibition halo of 1 cm (see Section 2).
[c]Numbers are the average of three independent experiments.

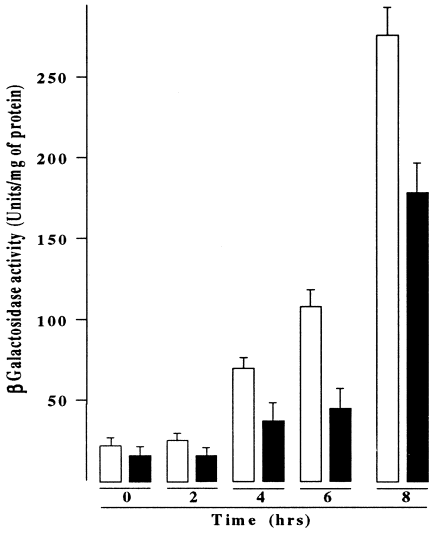

Fig. 4. Effect of α pheromone on *Fus1* induction in *ste4* cells carrying pGALHis constructs. Cells harboring pGALHis constructs and the Fus1-LacZ fusion into YEp351 were grown as indicated in Fig. 3. 1×10^7 Cells were transferred to 2 ml of SGal medium containing 1 μM of α pheromone. β-Galactosidase was determined at different times. Numbers correspond to the average of three independent experiments. Open bar, pGALHis-Ste4; Solid bar, pGALHis-ste4-10.

to **a** pheromone (Table 1). These data indicate that the low activity of ste4-10p mutant is due to defective activation of G protein by the pheromone-bound receptor rather than to impairment to activate the downstream effector.

Defective response of the *ste4-10* to sex pheromone could be due to either defective interaction of Ste4p with the Ste2 receptor or defective coupling of receptor to G protein as a result of an altered interaction between Ste4p and Gpa1p. To address this question we determined physical interactions between the wild type and mutant Ste4p subunits with both Gpa1p and the C-terminus of the Ste2 receptor employing the two-hybrid interaction assay system [25]. This system consists on a DNA binding domain plasmid (pEG202) that expresses in a constitutive manner a LexA-fused protein under the control of the *Adh1*promoter, and a transcription activation-domain plasmid (pJG4-5) where acid blob B42-fused proteins are expressed activating the *Gal1* inducible promoter. Employing this system, we determined in previous assays lack of physical interaction of Ste4p and Gpa1p with full length Ste2 receptor and fragments containing its three cytoplasmic loops (data not shown). However, both subunits associate at the same level with the AluI(269) fragment (Table 2) which corresponds to the inner portion of the C-terminus tail of Ste2p. The two-hybrid assay detected that while ste4-10p mutant interacts with the AluI(269) fragment as the wild type Ste4p, it has an association with Gpa1p that is 10 times weaker than the wild type subunit (Table 2). These data indicate that the ste4-10p subunit associates normally with the Ste2 receptor but has a defective interaction with Gpa1p.

Overexpression of the Gpa1p subunit completely inhibits growth arrest induced by overproduction of Ste4p subunit [21]. Taking this into account, we observed that Gpa1p-dependent inhibition of growth arrest follows the same kinetics in *ste2* cells, overexpressing either Ste4p or ste4-10p (not shown). This experiment indicated that the signalling function of Ste4p and ste4-10p can be blocked with the same intensity by the inactive form of Gpa1p.

Full length sequencing of the *ste4-10* allele, showed a single transversion of adenine 396 by cytosine that rendered the replacement of leucine 132 by phenylalanine. This was the only substitution found in the full length gene. Leu-132 lies at the end of the 1st blade of the proposed propeller structure of the subunit [4,5]. According to the crystal structure of the trimeric G protein it lies on the boundaries of the interacting regions of β with α subunit and receptor. Leu-132 is an almost fully conserved residue in the β subunits known so far. It maps close to some residues that in yeast have been indicated as contact sites with Gα, some of which when substituted disrupt Gβ-Gα interaction [4,32].

The results here described depict a picture where the single point mutation in *ste4-10* uncouples G protein from the pheromone-activated receptor by reducing association with Gpa1p subunit and point that leu-132 could have a central role in yeast β activity.

4. Discussion

The actual model for the receptor-Gαβγ complex specifies interactions between the receptor's third cytoplasmic loop, the Gα N- and C-termini, and the Gβ subunit, in addition to a contact between the receptor's C-terminal tail and the Gβγ dimer [33]. The regions of the yeast Gβ that interact with downstream signaling components have been identified genetically and map to the N-terminal coiled coil of Gβγ [34,35]. None of the residues on yeast Gβγ that are assumed to form part of the effector site lie within a Gα/Gβγ interface.

Table 2
Physical association of hybrid proteins[a]

DNA-binding domain[b]	Transcription activation domain[c]	β-Galactosidase activity[d] (U/mg/ml)$\times10^{-3}$
Cts1-2p	Ste4p	4.5 ± 1.0
Cts1-2p	Ste4-10p	4.0 ± 0.5
Cts1-2p	Gpa1p	5.5 ± 1.0
Ste2($AluI_{269}$)p	Ste4p	65.0 ± 8.0
Ste2($AluI_{269}$)p	Ste4-10p	66.0 ± 11.0
Ste2($AluI_{269}$)p	Gpa1p	70.0 ± 10.0
Gpa1p	Ste4p	1005.0 ± 40
Gpa1p	Ste4-10p	115.0 ± 21

[a]Expression of *LacZ* reporter gene (pS18-34) was induced by incubating EGY48 cells harboring plasmids in 5 ml SGal medium for 12 h at 30°C.
[b]DNA-binding domain refers to LexA-fused proteins cloned into pEG202.
[c]Transcription activation domain refers to B42-fused proteins cloned into pJG4-5.
[d]β-Galactosidase activity corresponds to the average value of three independent clones measured simultaneously.

L132 of the Ste4p subunit is a conserved residue (with few exceptions) that lays within the second WD-40 repeat [36]. The results here reported indicate that this residue can be important for proper functional coupling of receptor-G protein. Alteration of this residue modifies Ste4p-Gpa1p interaction without affecting downstream signaling activity of Ste4p, i.e. effector activation and Ste18p association. To our knowledge, this is the only mutation described so far that shows this striking phenotype. In contrast, mutations in W136 and or L138 disrupt interaction with Gpa1p but show a constitutive activation of the pheromone response pathway [32].

Based on crystal structures recently inferred for trimeric G protein [3,4] the L132 of the Ste4p subunit is located at the end of the first blade of the proposed propeller, in a region that makes physical contacts with the switch II region of the Gpa1p subunit [4,6]. The opposition of the two subunits in this region shields this vital domain, protecting the Gα face until the cationic lever of the receptor flips the switch domain and triggers the Gα conformational change, thus providing a mechanistic explanation for subunit dissociation. It has been proposed that a number of salt bridges and the fit of Gβ W99 (W136 in Ste4p) into a hydrophobic pocket on Gα determine binding of Gα to the top of the propeller (reviewed in [2]). In some way presence of phenylalanine in position 132 of the *ste4-10* mutant may have a disturbing effect in this interaction.

In summary, the Ste4p subunit of the G protein involved in the yeast mating pathway appears to has an essential role in the proper functional coupling between receptor and G protein.

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