The β Subunit of the Heterotrimeric G Protein Triggers the *Kluyveromyces lactis* Pheromone Response Pathway in the Absence of the γ Subunit

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The *Kluyveromyces lactis* heterotrimeric G protein is a canonical Gαβγ complex; however, in contrast to *Saccharomyces cerevisiae*, where the Gγ subunit is essential for mating, disruption of the Klgα gene yielded cells with almost intact mating capacity. Expression of a nonfarnesylated Gγ, which behaves as a dominant-negative in *S. cerevisiae*, did not affect mating in wild-type and ΔGγ cells of *K. lactis*. In contrast to the moderate sterility shown by the single ΔKlgα, the double ΔKlgα ΔKlgγ mutant displayed full sterility. A partial sterile phenotype of the ΔKlgγ mutant was obtained in conditions where the Klgβ subunit interacted defectively with the Gα subunit. The addition of a CCAAX motif to the C-end of Klgβ, partially suppressed the lack of both Klgα and Klgγ subunits. In cells lacking Klgγ, the Klgβ subunit cofractionated with Klgα in the plasma membrane, but in the ΔKlgα ΔKlgγ strain was located in the cytosol. When the Klgβ-Klgα interaction was affected in the ΔKlgγ mutant, most Klgβ fractionated to the cytosol. In contrast to the generic model of G-protein function, the Gβ subunit of *K. lactis* has the capacity to attach to the membrane and to activate mating effectors in absence of the Gγ subunit.

INTRODUCTION

Signal transduction mediated by heterotrimeric G proteins coupled to seven transmembrane receptors is an extremely widespread phenomenon in eukaryotic cells. In *Saccharomyces cerevisiae* the heterotrimeric Gα(Gpa1p)/Gβ(Gβ5p)/Gγ(Gγ18p) is required for response to mating pheromones. This Gγ protein is the same in MATα and MATα cells. On pheromone interaction with a cell type–specific receptor, the G protein dissociates into Gα(GTP) and the Gβ/Gγ dimer, which in turn initiates a cascade of events that results in transcriptional activation of genes required for mating (Elion et al., 1993, Olson et al., 2000). Mating between haploid cells of the opposite mating types leads to the formation of a diploid MATα/MATα cell. In *S. cerevisiae* disruption of the gene encoding the G-protein α subunit leads to permanent growth arrest and therefore to lethality (Dietzel and Kurjan, 1987; Miyajima et al., 1987), whereas inactivation of both, the Gβ and Gγ subunits leads to sterility (Whiteway et al., 1989). Moreover, Gβ subunit overexpression induces growth arrest and mating. On pheromone activation in *S. cerevisiae*, the liberated Gαβγ dimer directly associates with a scaffold protein Ste5p and with a p21-activated kinase (PAK), Ste20p, which is essential for activation of the MAPKKK Ste11p. Activation of Ste11p is also promoted by action of the adaptor protein Ste50p. Ste11p in turn, activates the MAPKK Ste7p. Downstream from Ste7p, Fus3p and Kss1p, two partially redundant MAPKs, induce the activation of transcription factors, Ste12p among others, which regulate the mating process (Breitkreutz et al., 2001).

In the budding yeast *Kluyveromyces lactis*, the signal transduction system that mediates mating is triggered by both Gα (Saviñón-Tejeda et al., 2001) and Gβ (Kawasaki et al., 2005) subunits of the heterotrimeric G protein. In contrast to *S. cerevisiae*, inactivation of Gα in *K. lactis* does not affect cell viability, but produces partial sterility (Saviñón-Tejeda et al., 2001); and overexpression of Gβ has no effect in mating, but its inactivation produces total sterility (Kawasaki et al., 2005). These features of *K. lactis* G protein function, not observed in *S. cerevisiae*, may reflect a different control mechanism of the process for sexual reproduction. The actual knowledge of the pheromone response pathway in *K. lactis* shows that activation of G protein by binding of pheromone to G protein–coupled receptor triggers two branches: one is essential for mating and is triggered by the Gβ subunit, and the second is dispensable and is activated by Gα. These two branches converge in the MAP module formed by the scaffold KISte5p, the MAPKK KISte11p, the MAPKKK KISte7p, and the MAPK KIFus3p (Kawasaki et al., 2008).

In the heterotrimeric G protein–coupled receptor systems, the βγ dimer is a fundamental part of the transduction mechanism. Yeast Gβ and Gγ form a stable dimeric complex similar in its structural and functional organization to the Gβγ-dimer of vertebrates. The Gβγ complex is associated with the membrane via isoprenyl modifications of the Gγ subunit and promotes Gα association with membranes and receptors (Zhang and Casey, 1996). The Gβγ dimer can also
activate effector proteins on its own or in parallel with Go subunit (Clapham and Neer, 1993; Neer, 1995).

Crystal structures of the mammalian Gβγ dimer have been solved (Sondek et al., 1996). The Gβ subunit has a β-propeller structure, containing seven so-called WD repeats, each repeat being one blade of the propeller. The crystal structure has shown that the Gγ subunit interacts with Gβ via an N-terminal coiled coil domain. Like Gβ of higher eukaryotes, the S. cerevisiae Gβ also has seven WD repeats of 23–41 residues flanked as a rule by Gly/His at the N-terminus and Trp/Asp at the C-terminus. The N-terminus of yeast Gβ extends ~89 residues and is capable of forming a regular amphipathic helix enabling it to participate in the formation of a stable dimer with the N-terminus of Gγ, which is itself a very long helix (Sondek et al., 1996).

Although most Gβγ dimers found in fungi species share the characteristics mentioned above, the Gβ subunit present in the fission yeast Schizosaccharomyces pombe that lacks the N-terminal extension still associates with the Gγ subunit Git11 (Landry and Hoffman, 2001), indicating that the WD repeat of the Gβ subunit is sufficient to allow assembly of the Gβγ dimer.

The Gβγ dimer in S. cerevisiae is the main transducer of the pheromone signal that promotes mating. The Gβγ dimer not only regulates positively the coupling between Go and the pheromone receptor (Blumer and Thorner, 1990), but also is required for the full activation of the Gγ subunit, which is itself a very long helix (Sondek et al., 1996). The Gγ subunit, together with Gβ, is responsible for triggering the pheromone pathway.

Even though K. lactis diverged from S. cerevisiae before the whole genome duplication (Scannell et al., 2007), they are considered close relatives. Most orthologous genes in both species are highly conserved, for example, the G protein α and β subunits show 72% (Savin˜o´n-Tejeda et al., 2000) and 63% (Kawasaki et al., 2005) similarity in their amino acid sequence, respectively. Contrary to the expectation that the Gβγ function should be conserved between the two species, in this work we present evidence that the Gβ subunit is capable of positively activating the K. lactis pheromone response pathway in the absence of the Gγ subunit.

MATERIALS AND METHODS

Strains and Media

Yeast strains used in this work were as follows: Kluveromyces lactis: 155 (MATα, ade2, his3, trp1, ura3-52, 2 mer-lox-ApH), was used for all hybrid assays (Codelis et al., 1997). Escherichia coli strain DH5α was used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% bacto-peptone, and 2% glucose. YPGal was the same except that it contained galactose instead of glucose. SD minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI) and 2% glucose. SGal was the same except for the substitution of glucose by galactose. For plasmid propagation, E. coli DH5α was used to propagate recombinant plasmids in bacteria. SD medium containing 2 mg/ml uracil and 1 mg/ml 5-fluoroorotic acid (5-FOA) was used for negative selection of the URA3 cassette.

Gene Disruptions

**KISTE18** (Gγ) gene disruptions in both MATα and MATa cells were achieved by homologous recombination and URA3 selection. A 366-base pair, PCR fragment (obtained with primers: 39 CTTTGTTTTCGTTTTTT - 25 [primer 1] and + 320 ATGAAATTCGTTAAT AAG + 306 [primer 2]) containing the full open reading frame (ORF) was ligated into the pGEM-T-Easy vector (Promega, Madison, WI) and then subcloned as a 365-base pair EcoRI frag-
Protein Interactions
Assays of physical interaction were done with the LexA-B42 two-hybrid system as described (Ongay-Larios et al., 2000). Cloning of GPA1 and STE4 from *K. lactis* into pEC202 and pJG4-5 were reported previously (Kawasaki et al., 2005). Cloning of ScSTE4 into plasmid pJG4-5 was reported previously (Ongay-Larios et al., 2000). To clone ScSTE18 into pEC202, a 341-base pair PCR fragment in which EcoRI and SalI restriction sites were introduced in positions −3 and +336 was ligated into pEC202 digested with the same enzymes. To clone STE4R313 into pJG4-5, plasmid pCEMSTE4R313 was digested with Xhol and NcoI. The 350-base pair fragment thus obtained was used to replace the wild-type fragment in pJG4-5STE4 with the same enzymes. Protein interaction was determined by expression of the C-termini of Gα subunits showing Gβγ dimers from both species. Amino acid residues are colored following the same pattern as the ribbon model, except for amino acids that make contact between the coiled-coil domains of Gβ and Gγ, which are shown in blue. Numbers in parentheses indicate the position of the last residue shown.

**Cell Fractionation**

Yeast cells were grown at 30°C in YPGal medium to midlog phase and harvested, washed twice with 50 mM Tris (pH 7.5), and then resuspended in 50 mM Tris, pH 7.5, 1 M sorbitol, and 5% β-mercaptoethanol. Spheroplasts were prepared by adding Lyticase (500 U/g cells) and PMSF (1 mM) and incubated for 24 h at 30°C for blue colony determination. Quantification of β-galactosidase activity was done as described (Ongay-Larios et al., 2000).

**Immunoblotting**

Proteins were resolved by SDS-PAGE, electrotransferred to nylon membranes (Millipore, Bedford, MA), and blocked in 5% skim milk in phosphate-buffered saline (PBS) + 0.05% Tween-20. Blots were incubated with rabbit anti-HA (Roche, Indianapolis, IN), anti-Hog or anti-Gpa1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies following the suppliers directions. Filter-bound antibodies were detected with HRP-conjugated secondary goat anti-rabbit IgG antibody (Zymed, South San Francisco, CA) and visualized with chemiluminescent HRP substrate (Millipore).

**Structural Modeling**

The Gβγ dimer and Gαβγ trimer from *K. lactis* were modeled taking the coordinates for the previously published Gα1β1γ2 trimer (Wall et al., 1995), whereas the S. cerevisiae models were taken from the RCSB Protein Databank database and checked with Molprobity (Lovell et al., 2003). Most modeling steps were done using Modeler 9v4 (Martí-Renom et al., 2000) except that sequence alignment was verified manually and the final model was generated by removing any structure exhibiting knots. Energy minimization was done with Chimera (Pettersen et al., 2004). Model structures were displayed using the PyMOL program (http://www.pymol.org/).

**RESULTS**

**A Typical Gγ Subunit Is Expressed in K. lactis**

In *S. cerevisiae*, the Gβγ dimer of the heterotrimeric G protein, mediates activation of the MAPK cascade that regulates the pheromone response pathway. Gβ and Gγ form a stable complex similar in its structure to the mammalian Gβγ dimer. *S. cerevisiae* Gβ is a propeller with seven blades and an extended N-terminal amphipathic helix, capable of forming an intermolecular complex with Gγ (Figure 1A). The N-terminal region of Gγ is itself a long regular helix that extends as a coiled-coil with the N-terminus of Gβ. Computer modeling of the *K. lactis* Gβ and Gγ subunits shows that they can adopt a similar structure as that of the *S. cerevisiae* Gβγ dimer (Figure 1A).
The *K. lactis* genome contains a single copy of the KISTE18 (KLLA 0E06138g) gene that encodes a typical γ subunit of heterotrimeric G proteins. It has significant homology to ScGγ (55% identity and 73% similarity, Figure 1B). KlGγ is 90 amino acids long and contains the conserved C-terminal CCAAX motif (CCTIM) that is a potential target for farnesylation at Cys^{87} and for palmitylation at the preceding Cys^{86} (Hirschman and Jenness, 1999). The presence of these highly conserved Cys residues in most fungal species suggests a conserved mechanism for the association of Gγ with the membrane. KlGγ contains an N-terminal α helical structure with heptad periodicity capable of forming a stable coiled-coil interaction with the long N-terminal region of the KIGβ subunit (Figure 1C). Most of the amino acid residues responsible for the formation of a stable Gβγ dimer complex are highly conserved in both KlGβ and the KlGγ subunits. Twelve of 15 amino acid residues thought to form the coiled-coil domain are identical in the Gγs of *K. lactis* and *S. cerevisiae*. Within this region, the amino acids that are predicted to make contact with the coiled-coil of Gβs are also identical (Figure 1C). The high similarity observed between the structures of KlGβγ and ScGβγ dimers could suggest that they may have similar function in the mating process; however, although KlGβ interacted with ScGγ and ScGβ interacted with KlGγ in a two hybrid experiment (see Figure 4), neither KlGβ (Kawasaki *et al.*, 2005) nor KlGγ suppressed the sterile phenotype displayed by *S. cerevisiae ΔGβ* and ΔGγ mutants respectively (not shown).

### The Gγ Subunit of the Heterotrimeric G Protein Is Dispensable for Mating in *K. lactis*

To test the role of the G-protein γ subunit in the pheromone response pathway in *K. lactis*, we isolated the KlGγ gene by PCR mediated amplification, and we introduced a URA3 cassette in the ORF generating at the same time a 61-base pair deletion. The Gγ gene was disrupted in both MATa and MATα cells by homologous recombination according to the strategy described in Materials and Methods. Southern blot analysis confirmed that URA3 transformants of both mating types carried the disrupted KlGγ allele. An expected HindIII 1.4-kb fragment that cross-reacted with the radiolabeled probe is observed in the wild-type strains, whereas two fragments are detected in the mutant loci due to an extra HindIII site present in the YIp352 integrating plasmid (Figure 2A). Additionally, total RNA subjected to Northern blot analysis showed that ΔKlGγ mutants of both mating types lack the ~0.4-kb fragment corresponding to the Gγ mRNA observed in wild-type cells (Figure 2B).

Mating in *K. lactis* is triggered by both KlGα and KlGβ subunits of the heterotrimeric G protein (Savinón-Tejeda *et al.*, 2001; Kawasaki *et al.*, 2005). When the gene encoding the KlGβ subunit was inactivated the cells became sterile (Figure 3, Table 1; Kawasaki *et al.*, 2005), whereas inactivation of the gene encoding the KlGα subunit diminished mating to ~10% (Figure 3, Table 1; Saviñon-Tejeda *et al.*, 2001). Surprisingly, disruption of KlGγ had no effect in the mating process, yielding cells with almost intact capacity to mate (90% of the control) when assayed in crosses with wild-type cells (Figure 3, Table 1). The same mating efficiency was observed in ΔΔKlGγ mutants of both MATα and MATα cells, indicating that this phenotype is independent of the mating type. A slight mating defect (~55% of the control) can be observed when both mating partners carry the disrupted KlGγ allele (Table 1). These findings indicate that the KlGγ subunit is dispensable for mating in *K. lactis* and are in contrast with the sterile phenotype displayed by *S. cerevisiae* strains where the Gγ gene has been deleted (Whiteway *et al.*, 1989). The above observations also indicate that pheromone signaling can be satisfied in *K. lactis* by action of KlGα and KlGβ alone and suggest that KlGβ is able to activate mating effectors in the absence of the KlGγ subunit.

### The ΔKlGγ Mutant Mates Less Efficiently When Competed with Wild-Type Cells

Although the KlGγ subunit is dispensable for the mating pathway, the slight reduction in mating of the ΔΔKlGγ strain

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**Figure 2.** (A) Disruption of the KlGγ gene. Cells were grown overnight in YPD medium, and genomic DNA from MATa (155) and MATα (12/8) wild-type strains and their ΔΔKlGγ mutants was obtained by a standard phenol-extraction protocol. DNA was digested with HindIII, subjected to Southern blot analysis and probed with the full radiolabeled Gγ gene. (B) Expression of the KlGγ gene. Cells were grown to midlog phase in YPD medium, harvested, and resuspended in water. Total RNA from wild-type and disrupted strains was extracted by the standard acidic-phenol protocol and was subjected to Northern blot analysis using a KlGγ gene probe. Large and small rRNA are indicated.

**Figure 3.** Effect of inactivation of G protein subunits on mating. Mating was done by replica-plating strain 155 (WT) or its mutants onto YPD plates containing a lawn of MATα cells (strain 12/8), followed by incubation overnight at 30°C. Diploid selection was done by replica-plating onto SD. Pictures were taken after 48-h incubation at 30°C. For strains carrying plasmids YEpKD-HA-STE4 (+HAGβ) and YEpKD-HA-STE4R^{+130} (+HAGβR^{+130}), mating was carried on YPGal plates.
could explain why this gene is conserved in K. lactis. To address this question, we conducted a mating experiment where ΔKIGγ cells were competed in mating with wild-type cells. For this we combined 0.5 x 10^6 mutant cells with the same number of wild-type cells, of the same mating type, and mated them to a tester strain. Diploids arose from the wild-type–wild-type cross were identified by the ura3 auxotrophy compared with the URA3 genotype of the ΔKIGγ–wild-type diploids. In these conditions it was observed that 70% of the diploids obtained were formed by wild-type cells, whereas only 30% were obtained from the ΔKIGγ mutant, indicating that cells lacking the KIGγ subunit are less efficient in diploid formation when competed with wild-type cells.

**Table 1. Mating efficiency of K. lactis mutant strains**

| Strains          | Mating efficiency |
|------------------|-------------------|
| WT(155)          | × WT (12/8)       |
| ΔGα              | × WT              | 0.01 |
| ΔGβ              | × WT              | 0.05 |
| ΔGγ              | × WT              | 0.92 |
| ΔGα ΔGγ          | × WT              | <0.001 |
| WT[ΔGγ]          | × ΔGγ             | 0.95 |
| ΔGγ[ΔGγ]<sup>ty</sup> | × WT     | 0.94 |
| ΔGγ[ΔGγ]<sup>ty</sup> | × WT     | 0.92 |
| ΔGα ΔGγ          | × WT              | <0.001 |

Numbers are relative to the mating efficiency of the wild-type cross (155 x 12/8) and are representative of three independent experiments. All strains are derived from strain 155 and were mated using wild-type 12/8 or mutant 12/8 ΔGγ as tester strains. All 155 isogenic strains carried the YEpKD vector, alone or with the indicated Gγ allele. Crosses were done by mixing 1 x 10^6 cells of each parent and incubating in YPGal overnight at 30°C. Cells were collected, diluted, and plated on SD medium until colonies appeared.

**Replacement of Cys by Ser at Position 87 in KIGγ Has No Effect on Mating in K. lactis**

In *S. cerevisiae*, substitution of the Cys residue in the C-terminal CAAAX motif of the Gγ subunit affects its farnesylation, and upon G protein activation leads to dissociation of the Gβγ dimer from the plasma membrane (Manahan et al., 2000). Cells carrying this Gγ mutant form are insensitive to pheromone and hence are unable to mate (Whiteway and Thomas, 1994). Furthermore, substitution of the Cys residue located in the CAAAX motif by Ser produces an dominant-negative phenotype, i.e., the GγS mutant subunit is capable of sequestering the Gβ subunit to form an unproductive dimer, inducing sterility in *S. cerevisiae*, even in the presence of the wild-type Gγ (Grishin et al., 1994). To investigate if the equivalent unfarnesylated version of *K. lactis* Gγ subunit behaves as dominant-negative, we substituted the Gγ residue at position 87 (Figure 1) by Ser and expressed this mutant form in wild-type and ΔKIGγ strains. In crosses with a wild-type tester strain, no effect on mating was observed when either KIGγ or KIGγS<sup>ty</sup> were expressed in wild-type or in ΔKIGγ haploid cells (Table 1). These observations indicate that the unfarnesylated KIGγ is inert for the mating system in *K. lactis*.

**Inactivation of KIGγ in the ΔKIGα Mutant Produces Sterility**

The above results suggest that the KIGβ subunit is capable of accessing its active location to trigger the mating pathway, even in the absence of the KIGγ subunit. This could be accomplished by its interaction with the KIGα subunit. The KIGα-mediated targeting of KIGβ would not be competed by KIGγS<sup>ty</sup>. If this assumption is correct, then KIGβ will be unable to activate the mating pathway in a cell devoid of both KIGα and KIGγ subunits, and thus inactivation of the KIGγ subunits should eliminate the remaining mating capacity of the ΔKIGα mutant. An experiment of diploid formation showed that the double ΔKIGαΔKIGγ mutant was totally sterile (Figure 3, Table 1). This result shows that in the double ΔKIGαΔKIGγ mutant the KIGβ subunit is not functional and indicates that in order to activate the mating pathway, KIGβ requires KIGα and/or KIGγ subunits.

**The KIGβ and KIGγ Subunits Interact in a Two-Hybrid Assay**

Although KIGβ and KIGγ have the structural requirements to form a stable complex (Figure 1), it is possible that in vivo these subunits do not interact with each other. To address this question, we determined physical interactions between wild-type KIGβ and KIGγ subunits by means of the two hybrid interaction system. The assay consisted on a DNA binding domain composed of a LexA-Gγ fusion protein under the control of the Adh1 promoter, and a transcription activation domain containing the acid Blob B42-Gβ fusion protein under the control of the Gal1 inducible promoter (Golemis et al., 1997). Determination of blue-colony intensity and quantification of β-galactosidase activity showed that KIGγ is able to associate strongly with the KIGβ subunit (Figure 4). The KIGβ-KIGγ interaction was as strong as the interaction observed between LexA-Gα with B42-Gβ fusions (Figure 4). As mentioned above, we also observed heterologous interaction between Gβ and Gγ subunits from *K. lactis* and *S. cerevisiae*, indicating that some of the basic elements leading to formation of Gβ/Gγ complexes are conserved in these two species.

**The KIGβ Subunit Is Located in the Membrane Fraction in the ΔKIGγ Strain**

Membrane anchoring of the Gαβγ complex is needed for efficient signaling in *S. cerevisiae*. For this, it has been shown that not only the Gγ subunit is farnesylated and S-palmitoylated but also the Gα subunit is N-myristoylated and S-palmitoylated (Dohlman and Thorner, 2001). Because no modification has been observed in the Gβ subunit of *S. cerevisiae*, its attachment to the membrane depends entirely on Gγ. To determine the cellular localization of the KIGβ subunit in the ΔKIGγ and the ΔKIGαΔKIGγ mutants in *K. lactis* we separated the cytosolic (C) and membrane (M) fractions from these strains and performed immunodetection on nylon membranes as described in Materials and Methods. To detect the KIGβ subunit we fused the HA epitope at its N-terminus (HAGβ) and probed it with an anti-HA antibody. To detect KIGα we probed the nylon membranes with an antibody against the *S. cerevisiae* Gpa1 protein, and finally, we used an anti-Hog1 antibody to detect Hog1p, a protein implicated in response to hyperosmotic stress that is located in the cytosol in iso-osmotic conditions (Westfall et al., 2008). As shown in Figure 5, the HAGβ subunit is detected in the membrane fraction when expressed in a strain lacking the endogenous KIGβ subunit. The HAGβ protein is predominantly associated to the membrane fraction in the
ΔKlGβΔKlGγ mutant, although a small but detectable amount is also present in the cytosolic fraction. In these two strains, the HAGβ cofractionates with the KlGα subunit, consistent with membrane localization of both proteins. However, when the HAGβ protein was expressed in the double ΔKlGαΔKlGγ mutant, it was detected only in the cytosolic fraction, colocalizing with Hog1p. These results strongly suggest that in the absence of KlGγ, the KlGβ subunit remained tethered to the plasma membrane by its association with the KlGα subunit. In the absence of both KlGα and KlGγ, the KlGβ subunit is released from the membrane and becomes nonfunctional. Although a small fraction of the HAGβ protein is located in the cytosol in the ΔKlGβΔKlGγ strain this is not the result of overexpression since HAGβ was never detected in the cytosolic fraction in the ΔKlGβ mutant; instead, we think that the KlGα-KlGβ association is not strong enough to titrate the full amount of HAGβ and/or the cellular content of KlGβ in this strain exceeds that of KlGα. These results are also in full agreement with the observations made in the mating experiments, where KlGβ triggers mating only in the presence of either KlGα or KlGγ or both proteins. Finally, the chimeric HAGβ protein retained its function since it is able to reverse the sterile phenotype displayed by the ΔKlGβ and ΔKlGβΔKlGγ mutants, but as expected, fails to reverse the sterility of the ΔKlGαΔKlGγ mutant (Figure 3).

Targeting of KlGβ Depends on KlGα in the Absence of KlGγ

To test the hypothesis that attachment of KlGβ to the plasma membrane is due to its interaction with KlGα when the KlGγ subunit is missing, we determined the cellular localization of a KlGβ subunit in which the Trp130 has been substituted by Arg (KlGβR130) and investigated the role of this mutant subunit in the mating pathway. It has been described that in S. cerevisiae the equivalent mutation in Gβ (Trp136 × Arg) diminishes its association with Gα without affecting its capacity to activate effectors and to trigger mating (Whiteway et al., 1994). This Trp residue is highly conserved among fungi Gβs, lying within the second WD motif and as deduced by the structural model of Gaβγ trimer, is located in a loop of the second blade of the β propeller, forming the interface that makes contact with Gα (Figure 6). According to the structural model, the Trp130 residue of the KlGβ subunit faces Ile278 and Glu280 of the Gβ subunit (K. lactis coordinates) and maintains a productive interaction with Glu280. These residues are also conserved in the S. cerevisiae Ga (Figure 6); therefore it is reasonable to assume that

![Figure 5. Immunodetection of the KlGβ subunit.](image)

![Figure 6. Ribbon representation of the Gaβγ complexes from K. lactis and S. cerevisiae.](image)
substitution of Trp130 by Arg in KlGβ will produce the effect described in *S. cerevisiae*. By PCR, we introduced Arg at position 130 in the HAGβ subunit and determined its localization by immunodetection with the anti-HA antibody. It was found that the HAGβR130 subunit cofractionated with KlGα in the membrane fraction when expressed in the ΔKlGβ mutant (Figure 5); however, when expressed in the double ΔKlGβΔKlGγ mutant it was mostly detected in the cytosol, although a significant amount was still detected in the membrane fraction. We concluded that the increased solubilization of HAGβR130 was the result of a defective interaction with the KlGα subunit; however, they still associate at a limited level. In a control two hybrid experiment for physical interaction, we observed that indeed, KIGβR130 interacted with KlGα 3-fold less efficiently compared with the interaction of KlGα with the wild-type KIGβ subunit (Figure 4). In agreement with the result found in *S. cerevisiae* (Whiteway et al., 1994), substitution of Trp130 by Arg in *K. lactis* Gβ, did not affect its interaction with the KlGγ subunit (Figure 4). Presence of both KlGα and KlGγ totally prevented the partition of HAGβR130 to the cytosolic fraction (Figure 5). However, the HAGβR130 was detected entirely in the cytosolic fraction when it was expressed in a strain devoid of both KlGα and KlGγ subunits, (Figure 5).

Finally, we determined the effect that the substitution of Trp130 by Arg in KIGβ has in the mating pathway of *K. lactis*. In agreement with the localization and interaction experiments, expression of HAGβR130 in the ΔKlGβ mutant restored mating to almost wild-type level, whereas mating of a strain devoid of KlGγ was significantly reduced (Figure 3). It was possible to observe an increased mating efficiency in the ΔKlGβΔKlGγ strain expressing HAGβR130 with long incubation periods of the mixture crosses, although it never reached the mating efficiency of cells expressing the wild-type KIGβ. These observations suggest that the formation of a small amount of KlGαKlGβR130 complex is enough to sustain limited diploid formation. As expected, HAGβR130 is unable to restore mating of the ΔKlGαΔKlGγ strain (Figure 3).

**Addition of a CCAAX Motif to KIGβ Bypasses Inactivation of KlGα and KlGγ Subunits**

The observation that a KIGβ subunit can remain functional in the absence of a KIGγ subunit when the KIGα protein is present indicates that KIGβ can fold properly and with the KIGα help, find its functional site at the plasma membrane. This would implicate that artificial anchoring of KIGβ to the membrane will eliminate the need for both KIGα and KIGγ. To test this hypothesis, we fused the coding region for the C-terminal seven residues from KIGγ (SACCTIM) to the C-terminus of the HAGβ subunit. The HAGβ-CCAAX chimeric protein suppressed, although at a very limited level, the mating defect of the ΔKlGαΔKlGγ double mutant (Figure 7A). This indicates that the addition of the CCAAX motif allows the KIGβ subunit to partially bypass lack of both KIGα and KIGγ subunits. Immunodetection of the HAGβ-CCAAX protein with the HA antibody showed that a significant proportion of the protein was attached to the plasma membrane, although most protein remained in the cytosolic fraction (Figure 7B).

**DISCUSSION**

*K. lactis* has two Gα subunits (KlGα1 and KlGα2), one Gβ, and one Gγ. Although KlGα2 is implicated in the regulation of cAMP (Savinón-Tejeda et al., 1996), KlGα1 and KlGβ are required for pheromone response (Savinón-Tejeda et al., 2001; Kawasaki et al., 2005). In this work, we investigated the role of the KIGγ subunit in the mating pathway of *K. lactis*, and we have found that ΔKlGγ mutants are fertile at near wild-type levels in crosses with wild-type cells. A slight defect in mating is observed only when the two mating partners lack the KIGγ subunit. Accordingly with the phenotype displayed by the ΔKlGγ mutant, substitution of the Cys residue of its CAAAX motif in KIGγ has no effect on mating in *K. lactis*. In contrast, it has been shown that the Gγ subunit is essential for mating in *S. cerevisiae* and that the substitution of the Cys residue of its CAAAX motif produces a dominant-negative phenotype (Grishin et al., 1994; Whiteway and Thomas, 1994). Therefore, for the mating pathway of *K. lactis*, it is clear now that the heterotrimeric G protein has a unique signaling mechanism (Figure 8). The three subunits positively control the mating process; however, although the KIGβ is essential in mating and lack of KIGα impairs mating significantly, the KIGγ subunit is practically dispensable. Thus, in this yeast species, KIGβ can activate mating in the presence of both subunits, or in the presence of either KIGα or KIGγ. Additionally, a KIGβ protein with reduced KIGα interaction can trigger mating efficiently in the presence of KIGγ, but with very limited capacity in its absence (Figure 8). All these observations indicate that the main contribution of KIGγ in the *K. lactis* signaling pathway is to enhance the membrane anchoring of KIGβ provided by KIGα.

In *S. cerevisiae*, both Gβ and Gγ are required for activation of downstream elements and inactivation of Gy prevents association of Gβ with the scaffold protein Ste5p (Whiteway et al., 1995). However our results indicate that in *K. lactis*, KIGβ can activate effector proteins in the absence of KIGγ. The fact that a heterotrimeric G protein can function as a heterodimer (GαGβ) supports the proposed model of G

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**Figure 7.** (A) Effect of the expression of Gβ-CCAAX on mating of ΔGαΔGγ mutant. Mating was done by replica-plating mutant strain ΔGαΔGγ (carrying YepKD alone or YepKD-HASTE4-CCAAX plasmid) onto YPGal plate containing a lawn of MATα cells (strain 12/8), followed by incubation overnight at 30°C. Diploid selection was done by replica-plating onto SD. Pictures were taken after 48-h incubation at 30°C. (B) Immunodetection of the KIGβ-CCAAX subunit. ΔGαΔGγ cells expressing the chimeric HAGβ-CCAAX protein under the control of the GAL1 promoter ( YepKD plasmid) were fractionated as indicated in Materials and Methods. Fifteen micrograms of protein from the membrane (M) or cytosolic (C) fractions was resolved in SDS-PAGE and analyzed by immunoblotting with either anti-HA or anti-Hog1.
protein subunits evolution, i.e., via the sequential addition of first Gβ and then Gγ subunit (Harashima and Heitman, 2002). However, mutant cells lacking Gγ are less mating competent when they have to compete with wild-type cells. The slight disadvantage in mating of ΔKlGγ mutants may exert selective pressure to maintain a Gγ gene in K. lactis.

Heterotrimeric G proteins functioning in mating have been described in other yeast species. For example, Candida albicans has two Ga subunits, one Gβ, and one Gγ. Although Ga2 is implicated in cAMP signaling and mating (Bennett and Johnson, 2006), Ga1 and the Gβγ dimer are required for the pheromone response pathway. Loss of either Ga1 or Gβ produces full sterility (Dignard et al., 2008), but effects of Gγ inactivation remain to be investigated. Cryptococcus neoformans contains three Ga subunits, one Gβ, and two Gγs. Ga1 regulates cAMP signaling (Alspaugh et al., 1997), whereas Ga2 and Ga3 have opposite roles in response to pheromones. Ga2 activates mating, whereas Ga3 inhibits mating (Hsueh et al., 2007). Inactivation of Gβ or Gγ totally eliminates mating, whereas Gγ1 inactivation diminishes it (Wang et al., 2000; Hsueh et al., 2007; Li et al., 2007). Overall, fungal species show a variety of mechanisms to activate mating using practically the same protein repertoire.

In the case of S. pombe the heterotrimeric G protein formed by Ga2, Gβ, and Gγ is not involved in mating but participates in the glucose sensing pathway and activates adenylate cyclase (Landry et al., 2000; Welton and Hoffman, 2000; Landry and Hoffman, 2001), whereas the pheromone signaling system is regulated by Ga1 alone and it seems that Gβγ has no role in this process (Ladds et al., 2005; Shpakov and Pertseva, 2008). Because deletion of KlGγ has no effect on mating in K. lactis and GγS87 seems to be inert, it could be that KlGβ and KlGγ do not form a dimeric complex. However, several observations indicate that these subunits can interact in vivo. First, the limited ability of the ΔKlGa mutant to form diploids is totally eliminated when KlGγ is inactivated, indicating that in the absence of KlGa, KlGγ is needed for the activation of KlGβ; second, mating efficiency drops to 50–60% when Gγ is disrupted in both mating partners, and third, KlGγ and KlGβ can physically associate in the two-hybrid assay. Moreover, analyses of KlGβγ dimers deduced by protein modeling, suggests that KlGβ and KlGγ conserve the structural features to form a dimer complex. Therefore we think that in K. lactis, the KlGβ and KlGγ subunits form a stable complex in vivo, but, nonetheless, this complex is not essential for KlGβ activation and mating response. A similar situation has been reported in S. pombe, where the Gβ subunit remains partially functional without a Gγ subunit; however the structure of these proteins present atypical features. The Gβ subunit Git5 lacks the N-terminal amphipathic helix present in most Gβ subunits (Landry et al., 2000; Shpakov and Pertseva, 2008), and the Gγ subunit Git11 has no N-terminal helix with the heptad periodicity capable of forming coiled-coil structures (Landry and Hoffman, 2001).

Other proteins with a similar structure to the Gβ subunits that regulate G protein function have been described. In S. pombe there is Gnr1p, a WD-40 repeat protein that adopts a structure similar to typical Gβ subunits. Gnr1p acts as a structural mimic of Gβ in the absence of a Gγ subunit. In S. cerevisiae there are the so-called kelch repeat proteins that lack the WD-40 repeat motifs but resemble the typical Gβ propeller. The kelch proteins Gbp1p and Gbp2p regulate cAMP signaling by inhibiting Ga2 activity (Harashima and Heitman, 2002).

Plasma membrane anchoring of the heterotrimeric G protein is a prerequisite for transduction of the pheromone stimulus to intracellular effectors in S. cerevisiae. To ensure membrane targeting of the G protein, the Ga subunit is N-myristoylated and S-palmitoylated, and the Gγ is farnesylated and S-palmitoylated (Dohlman and Thorner, 2001). Although inhibition of either Ga modifications in S. cerevisiae results in partial release of Gβγ, it has been observed that some Gβγ dimers remain attached to the membrane,
inducing constitutive signaling (Hirschman et al., 1997). On the other hand, inhibition of Gα farnesylation results in a sterile phenotype, whereas inhibition of GγS-palmitoylation significantly reduces its function (Whitehay and Thomas, 1994; Dohlman and Thorner, 2001). Additionally, association of Gβ to membranes is almost entirely dependent on the presence of Gγ (Hirschman et al., 1997), indicating that Gα is not sufficient for Gβ targeting in S. cerevisiae. Thus, a striking observation made in this work is that the lack of KlGγ has no apparent effect on the signal transduction during pheromone response in K. lactis as long as a functional KlGα is present, indicating that in this system, KlGβ can be targeted to the membrane by its interaction with KlGα. At present we do not know why this is different in S. cerevisiae; however, analysis of the interaction surfaces between Gα and Gβ deduced from the structural models of both species indicates that the hydrophobic interaction area in the K. lactis Gα/Gβ complex is 1270 Å² larger than that of S. cerevisiae. This may suggest that the Gα/Gβ interface is more stable in K. lactis. This assumption is supported by the following observation: the substitution of Trp136 by Arg in Gβ of S. cerevisiae created an allele that produces a haploid lethal phenotype (Hpl), and no compensatory mutations in Gα were found that suppressed the lethality induced by GβR136, suggesting that this mutant Gβ has lost complete interaction with Gα (Whitehay et al., 1994), whereas the substitution of the equivalent residue (Trp130) in K. lactis diminished Gα-Gβ interaction, but did not totally eliminate it. Modeling of the Gβ subunits of S. cerevisiae and K. lactis with Arg instead of Trp at positions 136 and 130, respectively, indicates that in both cases, the side chain of the Arg residue is too large to fit in the space occupied by Trp. This is enough to disrupt the interaction between Gα and Gβ in S. cerevisiae but not in K. lactis, confirming that the Gα-Gβ complex in K. lactis is more stable. Further studies will be required to find out which structural features make the Gα–Gβ interaction stronger in K. lactis, making the Gγ subunit dispensable for the pheromone response pathway.

It has been found that the dimeric Gβγ complex in S. cerevisiae is located mostly in plasma membranes, but a significant proportion associates with internal cell membranes and with the cytosolic fraction (Hirschman et al., 1997). The differential distribution of the Gβγ dimer could be explained by the existence of intermediates in the assembly and/or trafficking itinerary (Michaelson et al., 2002). However, in mutant cells devoid of Gγ, Gβ fails to associate with any cell membrane, becomes unstable, and is rapidly degraded; whereas inactivation of the Gα subunit diminishes Gβ association with the plasma membrane (Schmidt and Neer, 1991; Hirschman et al., 1997). In contrast, we have found that the KlGβ subunit is associated with membranes in K. lactis cells devoid of the KlGγ subunit, and when the KlGα is also eliminated, KlGβ fails to sediment with membranes and fractionates with the cytosol. Although our preparation is highly enriched with plasma membrane, we did not differentiate between pools of Gβγ associated to different membrane fractions, and we did not measure protein stability and turnover; thus, we were unable to determine to what extent, if any, the Gβ is altered in its stability and trafficking in the mutant strains. However, we expressed KlGβ from a plasmidic vector with constant promoter induction in our mutant strains, and we assume that most of the KlGβ produced should be correctly targeted in the plasma membrane in the ΔKlGβΔKlGγ cells because they mated at almost wild-type level.

Interestingly, an artificial KlGβ subunit containing the CCAAX motif from the Gγ subunit is able to attach to the membrane and stimulates the mating pathway even in the absence of both Gα and Gγ subunits, although at very limited level (Figure 8). The impaired signaling activity of Gβ-CCAAX may be due to conformational constraints on the protein that affect proper folding, to deficient coupling with the receptor and hence improper activation of the Gβ protein, and/or a deficiency association with effector proteins, such as Ste20p or Ste5p.

In conclusion, we have described that the Gβ subunit activates a signaling system in the absence of a canonical Gγ protein in the ΔKlGγ mutant which demonstrates that, in K. lactis, the formation of a Gβγ complex is not required to activate the mating cascade, which is distinct from the established paradigm of Gβγ function. However, this does not rule out the need of a Gβγ dimer to regulate other, yet unknown, signaling pathways in K. lactis.

The observation that the mating pathway in K. lactis can operate without a canonical Gβγ dimer may constitute a landmark in the evolution of G protein–signaling systems. It will be of utmost interest to determine whether this is the case in closely related yeast species or is a feature confined only to K. lactis.

The prominent role that S. cerevisiae has played in all research areas has led to the incorrect use of the term yeast as being synonymous with S. cerevisiae. However, yeast species differ in many properties such as morphology, carbon and nitrogen metabolism, regulation of fermentation and respiration, and—as has been shown in this work—sexual reproduction. Therefore, some paradigms emerging from S. cerevisiae should not be universally applied for all yeast species.

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