Molecular Biology of the Cell
Vol. 21, 489–498, February 1, 2010

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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Submitted June 10, 2009; Revised November 24, 2009; Accepted December 2, 2009
Monitoring Editor: Charles Boone

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 KlGγ 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 Δ*Gγ* cells of *K. lactis*. In contrast to the moderate sterility shown by the single Δ*KlGα*, the double Δ*KlGα* Δ*KlGγ* mutant displayed full sterility. A partial sterile phenotype of the Δ*KlGγ* 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 Δ*KlGα* Δ*KlGγ* strain was located in the cytosol. When the KlGβ-KlGα interaction was affected in the Δ*KlGγ* 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 heterotrimer Gα(Gpa1p)/Gβ(Ste4p)/Gγ(Ste18p) is required for response to mating pheromones. This G protein is the same in *MAT***a** 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 *MAT***a**/*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 Gβγ 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 KlSte5p, the MAPKKK KlSte11p, the MAPKK KlSte7p, and the MAPK KlFus3p (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

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–06–0472) on December 16, 2009.

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activate effector proteins on its own or in parallel with Gα 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 *Sschizosaccharomyces 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 Gα and the pheromone receptor (Blumer and Thorner, 1990), but also is the main activator of the effector proteins, such as Ste20p, a member of the p21-activated protein kinase (PAK) family (Leberer *et al.*, 2000); Ste5p, which is the scaffold of the MAP kinase module (Whiteway *et al.*, 1995; Inouye *et al.*, 1997); and Cdc24p, a guanine nucleotide exchange factor (GEF) for the small GTPase Cdc42 (Zheng *et al.*, 1994). As mentioned, lack of a Gγ subunit renders a Gβ subunit incapable of 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% (Saviñón-Tejeda *et al.*, 2001) 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: *Kluyveromyces lactis*: 155 (*MAT***a**, *ade2, his3, ura3*) and 12/8 (*MATα, lysA, argA, ura3*). *S. cerevisiae* strain EGY48 (*MATα, his3, trp1, ura3-52, leu2::pLeu-LexAop6*) was used for two-hybrid assays (Golemis *et al.*, 1997). *Escherichia coli* strain DH5α was used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% bactopeptone, 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 selection, SD minimal medium was supplemented with the required amino acids and nitrogen bases (50 μg/ml). LB plus ampicillin (100 μg/ml) 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

KlSTE18 (Gγ) gene disruptions in both *MATα* and *MAT***a** cells were achieved by homologous recombination introducing the *URA3* marker. A 360-base pair PCR fragment (obtained with primers: −39 CCTTGGTGTAGTTTT −25 [primer 1] and +320 ATGTAACTTAATAAG +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 fragment into the YIp352 integrative vector opened with the same enzyme. This integrative plasmid was linearized with BstEII and BglII (natural restriction sites in *KlSTE18*) and used for yeast transformation. *URA3* strains were selected and gene disruption was confirmed by Southern blotting. Disruption of *KlGPA1* (Gα) and *KlSTE4* (Gβ) genes was previously described (Saviñón-Tejeda *et al.*, 2001; Kawasaki *et al.*, 2005). All single mutants were grown on FOA for selection of *ura3* strains. Δ*Klgpa1*Δ*Klste18 and* Δ*Klste4*Δ*Klste18* double mutants were obtained by crossing single mutants and segregating double recombinants. The *Klste4* mutant was transformed with a plasmidic *KlSTE4* copy to alleviate its sterility before crossing it with *Klste18*. The double mutants were confirmed by Southern blot hybridization.

Gene Constructions and Mutagenesis

KlSTE18 was amplified by PCR from genomic DNA employing forward oligonucleotide + 1 ATGGGAGAGTTACCAT + 16 (primer 3), and backward primer 2 generating a 320-base pair product. This product containing the full *KlSTE18* ORF was subcloned into pGEM-T-Easy. A 332-base pair NcoI- SalI fragment (filled in with Klenow) was obtained from the pGEM-T-Easy clone and was ligated into YEpKD (Saviñón-Tejeda *et al.*, 2001) opened at EcoRI (filled in with Klenow), yielding YEpKDSTE18, which places the *KlSTE18* gene under the control of the *Gal1* promoter. To generate $G\gamma S^{87}$, YEpKDSTE18 was used as template in a PCR reaction employing primer 3 and oligonucleotide + 273 ATCACATTATGGTACTACTGGCACTCG + 247 (primer 4), which introduces A instead of T at position + 258 (underlined), rendering Ser instead of the Cys in position 87 of Gγ. The PCR product which contains the full *KlSTE18* ORF, was subcloned into pGEM-T-Easy and then into YEpKD following the same strategy as the wild-type gene. This generated $YEpKDSTE18S^{87}$.

KlSTE4 was cloned into YEpKD as previously described (Kawasaki *et al.*, 2005). A hemagglutinin (HA) epitope was introduced in frame into the N-terminus of KlSte4p as follows: The 3× HA epitope was PCR amplified using forward primer 5′-CCCGGGATGTACCCATACGATGTTCCTG-3′ (primer 5) and reverse primer 5′-*TGCTGAATCGACTTCCAT*AGCGTAATCTGGAACG-3′ using a plasmid containing three copies of the sequence encoding the HA epitope as template. The forward primer was designed with an SmaI restriction site (underlined) and the reverse primer overlaps with six codons of the *KlSTE4* 5′sequence (italics). This PCR product was then used as forward primer along with the *KlSTE4* reverse primer 5′-TTTTTCGATATGCGGTACCATTCTCAGT-3′, which contains an Asp718 restriction site (underlined), using plasmid YEpKDSTE4 as template. The 1434-base pair PCR product was ligated into pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA) to generate pTOPOSTE4. This plasmid was then digested with SmaI and Asp718, and the latter site was Klenow filled and the fragment thus obtained was cloned into YEpKDGalHis (Kawasaki *et al.*, 2005) previously digested with EcoRI and filled in with Klenow, rendering YEpKD-HA-STE4.

A 366-base pair fragment of *KlSTE4* was amplified by PCR employing the forward primer 6: +35 ACAGGAACTGATTGTGC + 51 and the mutagenic primer 7: +401 GCTACTCAAGACCCGTTGGGATAACAA + 375, which introduces a C instead of T at position + 387 (underlined). This yields the substitution of Trp130 by Arg. The PCR product was used as a forward primer in a second PCR reaction using backward primer + 704 ATTATGACCATATCG + 690 (primer 8). The product was cloned into pGEM-T-Easy generating $pGEMSTE4R^{130}$. A 369-base pair NdeI-NcoI restriction fragment was obtained from the $pGEMSTE4R^{130}$ plasmid and ligated into YEpKD-HA-STE4 digested with the same enzymes. This generated $YEpKD\text{-}HASTE4R^{130}$.

Addition of a CCAAX motif into the COOH-end of the Gβ subunit was done as follows: A PCR reaction was performed employing primer 5 and backward primer 5′-CCCGGG*TCACATTATGGTACAACAGGCACT*CTGATGAACC-GGTGT-3′, which contains an SmaI site (underlined), the last 24 nucleotides (italics) of the *KlSTE18* gene including its stop codon, and the last 15 coding nucleotides of the *KlSTE4* gene. A standard PCR reaction was performed using the plasmid YEpKD-HA-STE4 as template. The resulting PCR product encodes the HA-Gβ protein with the last seven amino acid residues (SACCTIM) from Gγ fused in its C-end. The PCR product was cloned into pGEM-T-Easy producing pGEMHA-STE4-CAAX. A SmaI fragment containing the fused gene was excised from this plasmid and subcloned into YEpKDGal previously digested with EcoRI and blunt ended with Klenow enzyme. This generated YEpKD-HA-STE4-CAAX.

Mating Assays

A cell patch of the strain to be tested was grown on YPD or SD (for strains carrying plasmids) for 24 h. The tester strain was grown as a lawn on YPD for 24 h. Both strains were replica plated onto an YPD or YPGal (for YEpKDGal constructs) plate and incubated overnight at 30°C to allow cells to mate. Diploids were selected at 30°C by replica plating on SD medium and photographed 48 h later. For quantitative mating assays, strains to be tested were grown until midlog phase in SD medium (for strains carrying plasmids) and mixed in 100 μl YPGal at a number of 1×10^6 cells each strain. Mating was carried out incubating overnight at 30°C. Suspensions were diluted and plated on SD medium until diploid colonies appeared. Mating competition experiments were carried out following the same recipe, except that strains to be tested were mixed (0.5×10^6 cells each) before mating them with the tester strain.

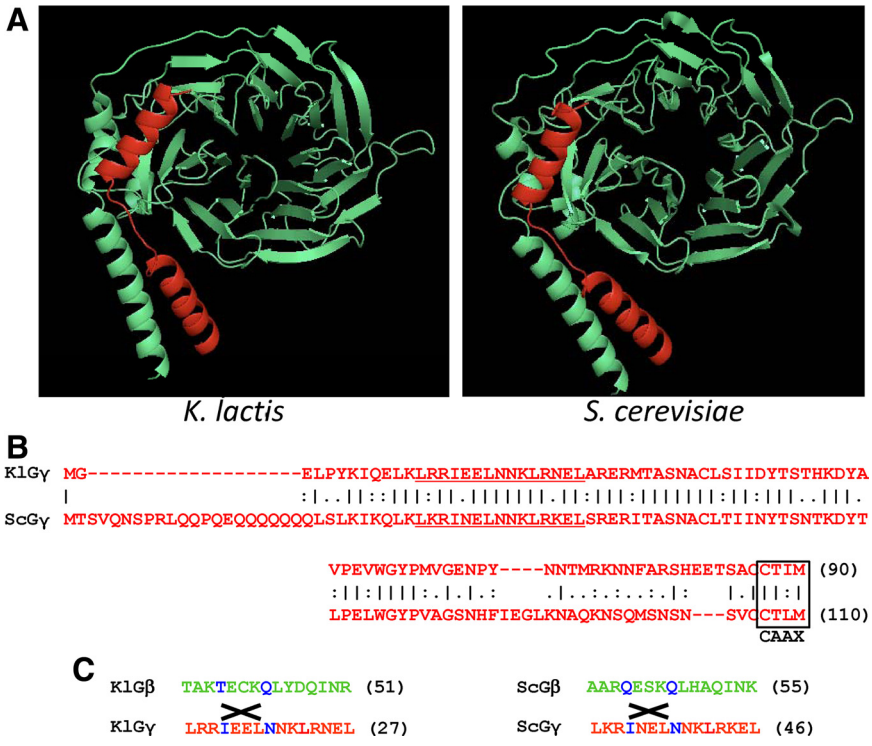

Figure 1. (A) Ribbon representation of the Gβγ dimers from *K. lactis* and *S. cerevisiae*, showing Gβ in green and Gγ in red. Regions not modeled due to disorder are not shown. (B) Alignment of Gγs with identical (|), conserved (:), and nonconserved (.) residues. The CAAX motif in the C-termini of Gγs is boxed. (C) Residues forming the coiled-coil domain in Gβ and Gγ 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.

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 pEG202 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 gene into pEG202, a 341-base pair PCR fragment in which EcoRI sites were introduced at position −3 and +336 was ligated into pEG202 digested with the same enzyme. To clone STE4R^{130} into pJG4-5, plasmid pGEMSTE4R^{130} was digested with XhoI and NcoI. The 350-base pair fragment thus obtained was used to replace the wild-type fragment in pJG4-5STE4 digested with the same enzymes. The *KlSTE18* gene was subcloned into pEG202 as an in frame 313-base pair PCR product in which EcoRI and SalI restriction sites were introduced in positions −3 and +315, respectively. For this, the pEG202 plasmid was opened with the same enzymes. Protein interaction was determined by expression of the *LACZ* reporter located in the pSH18–34 plasmid (Ongay-Larios *et al.*, 2000). *S. cerevisiae* endochitinase gene (*CTS1*) cloned into pEG202 (Ongay-Larios *et al.*, 2000) was used as negative interaction control. Strain EGY48 was transformed with two-hybrid plasmids and grown on SD plate at 30°C until colonies appeared. Selected clones were streaked on SGal medium containing 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; pH 7.0) 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).

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 120 min at 30°C. From this point on all steps were carried out on ice. Spheroplasts were sonicated four times for 1 min at 30-s intervals. The spheroplast suspension was centrifuged 10 min at 3000 ×*g* to remove unbroken cells and cell debris. The cleared lysate was centrifuged at 100,000 × g for 30 min. An aliquot of the supernatant (cytosol fraction) was saved to measure protein concentration, the rest was transferred to a clean tube and diluted with an equal volume of 2× SDS-PAGE sample buffer and boiled for 10 min. The pellet was resuspended in 50 mM Tris, pH 7.5, 1 mM EGTA (EGTA-Tris buffer) and centrifuged again 100,000 × *g* for 45 min. The pellet (membrane fraction) was resuspended in EGTA-Tris buffer. An aliquot was removed for protein quantification; the rest was kept at −70°C. Before electrophoresis, an aliquot of the membrane fraction was diluted with one volume of 2× SDS-PAGE sample buffer and boiled for 10 min.

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 Giα1β1γ2 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 (Marti-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/).

Other Methods

All recombinant genes and PCR products were sequenced in full. Probes for Southern and Northern blot analysis were labeled with [α-^{32}P]dCTP by the Random Prime Labeling System (Rediprime II, Amersham Biosciences, Piscataway, NJ). Standard Southern and Northern blot analysis, recombinant DNA technology, and yeast genetics procedures were performed in this work.

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 *KlSTE18* (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 palmitoylation 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 KlGβ 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* Δ*G*β and Δ*G*γ 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 *KlG*γ 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 *MAT*a and *MAT*α 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 *KlG*γ 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 Δ*KlG*γ 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 (Saviñón-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; Saviñón-Tejeda *et al.*, 2001). Surprisingly, disruption of *KlG*γ 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 Δ*KlG*γ mutants of both *MAT*a and *MAT*α 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 *KlG*γ 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.

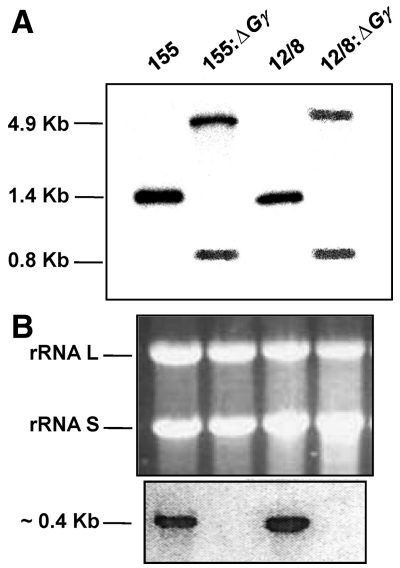

Figure 2. (A) Disruption of the *KlG*γ gene. Cells were grown overnight in YPD medium, and genomic DNA from *MAT***a** (155) and *MAT*α (12/8) wild-type strains and their Δ*KlG*γ mutants was obtained by a standard phenol-extraction protocol. DNA was digested with HindIII, subjected to Southern blot analysis and probed with the full radiolabeled Gγ gene. (B) Expression of the *KlG*γ 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 *KlG*γ gene probe. Large and small rRNA are indicated.

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 Δ*KlG*γ strain

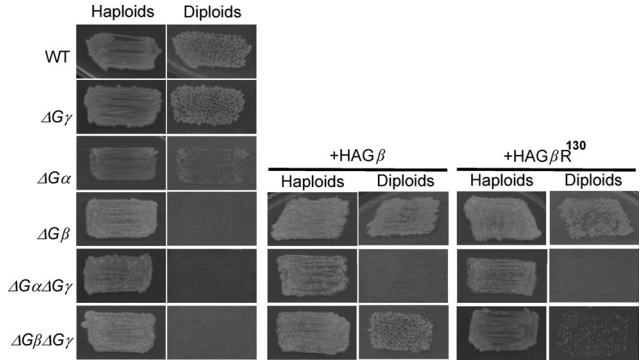

Figure 3. Effect of inactivation of G protein subunits on mating. Mating was done by replica-plating strain 155 (WT) or its mutants onto YPD plates containing a lawn of *MAT*α cells (strain 12/8), followed by incubation overnight at 30°C. Diploid selection was done by replica-plating onto SD. Pictures 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.

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

Strains			Mating efficiency
WT(155)	×	WT (12/8)	1.00
ΔGα	×	WT	0.10
ΔGβ	×	WT	<0.001
ΔGγ	×	WT	0.90
ΔGγ	×	ΔGγ	0.55
WT[Gγ]	×	WT	0.95
ΔGγ[Gγ]	×	WT	0.92
WT[GγS^{87}]	×	WT	0.94
ΔGγ[GγS^{87}]	×	WT	0.92
ΔGα ΔGγ	×	WT	<0.001

Numbers are relative to the mating efficiency of the wild-type cross (155 × 12/8) and are representative of three independent experiments. All mutants 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 1×10^6 cells of each parent and incubating in YPGal overnight at 30°C. Cells were collected, diluted, and plated on SD medium until colonies appeared.

could explain why this gene is conserved in *K. lactis*. To address this question, we conducted a mating experiment where Δ*KlGγ* cells were competed in mating with wild-type cells. For this we combined 0.5×10^6 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* auxotropy compared with the *URA3* genotype of the Δ*KlGγ*–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 Δ*KlGγ* mutant, indicating that cells lacking the KlGγ subunit are less efficient in diploid formation when competed with wild-type cells.

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

In *S. cerevisiae*, substitution of the Cys residue in the C-terminal CAAX 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 CAAX motif by Ser produces a 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 Cys residue at position 87 (Figure 1) by Ser and expressed this mutant form in wild-type and Δ*KlGγ* strains. In crosses with a wild-type tester strain, no effect on mating was observed when either KlGγ or KlGγS^{87} were expressed in wild-type or in Δ*KlGγ* haploid cells (Table 1). These observations indicate that the unfarnesylated KlGγ is inert for the mating system in *K. lactis*.

Inactivation of KlGγ in the ΔKlGα *Mutant Produces Sterility*

The above results suggest that the KlGβ subunit is capable of accessing its active location to trigger the mating pathway, even in the absence of the KlGγ subunit. This could be accomplished by its interaction with the KlGα subunit. The KlGα-mediated targeting of KlGβ would not be competed by KlGγS^{87}. If this assumption is correct, then KlGβ will be unable to activate the mating pathway in a cell devoid of both KlGα and KlGγ subunits, and thus inactivation of the KlGγ subunits should eliminate the remaining mating capacity of the Δ*KlGα* mutant. An experiment of diploid formation showed that the double Δ*KlGα*Δ*KlGγ* mutant was totally sterile (Figure 3, Table 1). This result shows that in the double Δ*KlGα* Δ*KlGγ* mutant the KlGβ subunit is not functional and indicates that in order to activate the mating pathway, KlGβ requires KlGα and/or KlGγ subunits.

The KlGβ and KlGγ Subunits Interact in a Two-Hybrid Assay

Although KlGβ and KlGγ 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 KlGβ and KlGγ 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 *Gal*1 inducible promoter (Golemis *et al.*, 1997). Determination of blue-colony intensity and quantification of β-galactosidase activity showed that KlGγ is able to associate strongly with the KlGβ subunit (Figure 4). The KlGβ-KlGγ 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 KlGβ Subunit is Located in the Membrane Fraction in the ΔKlGγ *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 KlGβ subunit in the Δ*KlGγ* and the Δ*KlGα*Δ*KlGγ* 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 KlGβ subunit we fused the HA epitope at its N-terminus (HAGβ) and probed it with an anti-HA antibody. To detect KlGα 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 KlGβ subunit. The HAGβ protein is predominantly associated to the membrane fraction in the

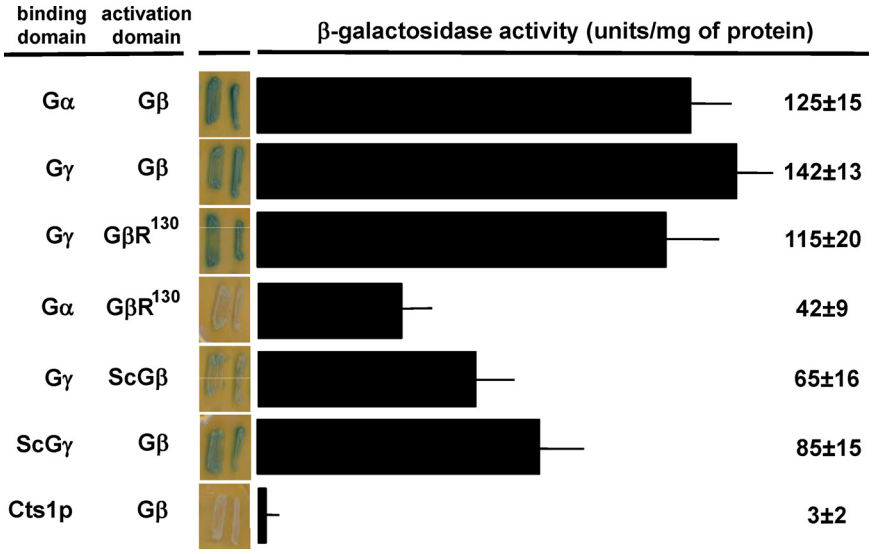

Figure 4. Physical association of G protein subunits determined by the two-hybrid system. The binding domain corresponds to LexA-fused proteins cloned into pEG202 and activation domain corresponds to B42-fused proteins cloned into pJG4-5. Gβ and Gγ from *S. cerevisiae* are named as Sc. Two-hybrid plasmids were introduced into strain EGY48, and two independent clones were plated on SGal plates containing 1 mg/ml X-Gal. Pictures were taken 24 h after incubation at 30°C. Quantitation of β-galactosidase activity was determined as described (Ongay-Larios *et al.*, 2000). The β-galactosidase activity corresponds to the average value of three independent clones. The *S. cerevisiae* endochitinase (Cts1p) was used as negative interaction control.

Δ*KlGβ*Δ*KlGγ* 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 Δ*KlGα*Δ*KlGγ* 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 Δ*KlGβ*Δ*KlGγ* strain this is not the result of overexpression since HAGβ was never detected in the cytosolic fraction in the Δ*KlGβ* mutant; instead, we think that the KlGα-KlGβ association is not strong enough to titer the full amount of KlGβ 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 Δ*KlGβ* and Δ*KlGβ*Δ*KlGγ* mutants, but as expected, fails to reverse the sterility of the Δ*KlGα*Δ*KlGγ* 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 (KlGβR^{130}) 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 Gαβγ 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 Gα subunit (*K. lactis* coordinates) and maintains a productive interaction with Glu280. These residues are also conserved in the *S. cerevisiae* Gα (Figure 6); therefore it is reasonable to assume that

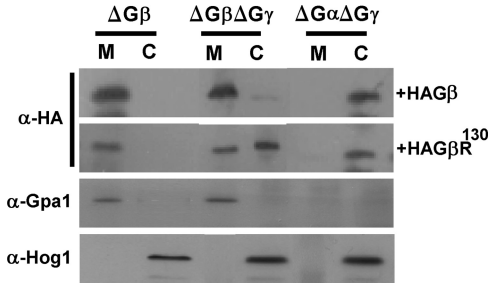

Figure 5. Immunodetection of the KlGβ subunit. Cells expressing the wild type (HAGβ) and mutant (HAGβR^{130}) HA epitope-tagged versions of KlGβ under the control of the *GAL1* promoter (YEpKD plasmid) were fractioned as indicated in *Materials and Methods.* Fifteen micrograms of protein from the membrane (M) or cytosolic (C) fractions was resolved in SDS-PAGE and analyzed by immunoblotting with either anti-HA, anti-Gpa1 (Gα subunit), or anti-Hog1.

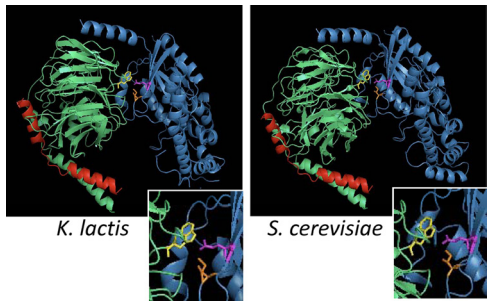

Figure 6. Ribbon representation of the Gαβγ complexes from *K. lactis* and *S. Cerevisiae*. Gα is shown in blue, Gβ in green, and Gγ in red. Regions not modeled due to disorder are not shown. The *K. lactis* Trp130 (136 in *S. cerevisiae*) in Gβ is shown in yellow, relative to Ile278 (magenta) and Glu280 (orange) of the Gα subunit (positions 303 and 305, respectively, in *S. cerevisiae*).

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 HAGβR^{130} subunit cofractionated with KlGα in the membrane fraction when expressed in the Δ*KlGβ* mutant (Figure 5); however, when expressed in the double Δ*KlGβ*Δ*KlGγ* mutant it was mostly located in the cytosol, although a significant amount was still detected in the membrane fraction. We concluded that the increased solubilization of HAGβ^{R130} 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, KlGβR^{130} interacted with KlGα 3-fold less efficiently compared with the interaction of KlGα with the wild-type KlGβ 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 HAGβR^{130} to the cytosolic fraction (Figure 5). However, the HAGβR^{130} 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 KlGβ has in the mating pathway of *K. lactis*. In agreement with the localization and interaction experiments, expression of HAGβR^{130} in the Δ*KlGβ* 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 Δ*KlGβ*Δ*KlGγ* strain expressing HAGβR^{130} with long incubation periods of the mixture crosses, although it never reached the mating efficiency of cells expressing the wild-type KlGβ. These observations suggest that the formation of a small amount of KlGαKlGβR^{130} complex is enough to sustain limited diploid formation. As expected, HAGβR^{130} is unable to restore mating of the Δ*KlGα*Δ*KlGγ* strain (Figure 3).

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

The observation that a KlGβ subunit can remain functional in the absence of a KlGγ subunit when the KlGα protein is present indicates that KlGβ can fold properly and with the KlGα help, find its functional site at the plasma membrane. This would implicate that artificial anchoring of KlGβ to the membrane will eliminate the need for both KlGα and KlGγ. To test this hypothesis, we fused the coding region for the C-terminal seven residues from KlGγ (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 Δ*KlGα*Δ*KlGγ* double mutant (Figure 7A). This indicates that the addition of the CCAAX motif allows the KlGβ subunit to partially bypass lack of both KlGα and KlGγ 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).

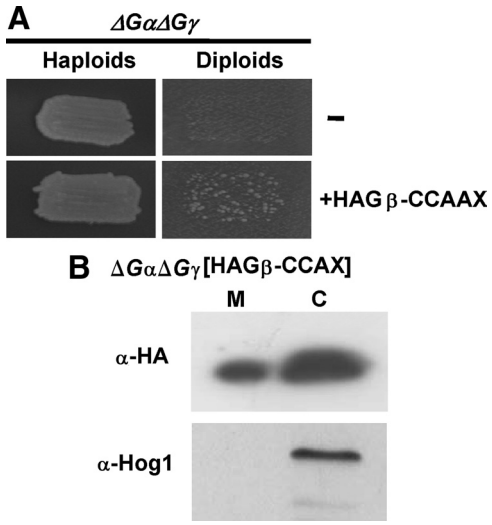

Figure 7. (A) Effect of the expression of Gβ-CCAAX on mating of ΔGαΔGγ mutant. Mating was done by replica-plating mutant strain ΔGαΔGγ (carrying YEpKD alone or YEpKD-HASTE4-CCAAX plasmid) onto YPGal plate containing a lawn of *MATα* cells (strain 12/8), followed by incubation overnight at 30°C. Diploid selection was done by replica-plating onto SD. Pictures were taken after 48-h incubation at 30°C. (B) Immunodetection of the KlGβ-CCAAX subunit. ΔGαΔGγ cells expressing the chimeric HAGβ-CCAAX protein under the control of the *GAL1* promoter (YEpKD plasmid) were fractioned 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.

DISCUSSION

K. lactis has two Gα subunits (KlGα1 and KlGα2), one Gβ, and one Gγ. Although KlGα2 is implicated in the regulation of cAMP (Saviñón-Tejeda *et al.*, 1996), KlGα1 and KlGβ are required for pheromone response (Saviñón-Tejeda *et al.*, 2001; Kawasaki *et al.*, 2005). In this work, we investigated the role of the KlGγ subunit in the mating pathway of *K. lactis*, and we have found that Δ*KlGγ* 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 KlGγ subunit. Accordingly with the phenotype displayed by the Δ*KlGγ* mutant, substitution of the Cys residue of the CAAX motif in KlGγ 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 CAAX 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 KlGβ is essential in mating and lack of KlGα impairs mating significantly, the KlGγ subunit is practically dispensable. Thus, in this yeast species, KlGβ can activate mating in the presence of both subunits, or in the presence of either KlGα or KlGγ. Additionally, a KlGβ protein with reduced KlGα-interaction can trigger mating efficiently in the presence of KlGγ, but with very limited capacity in its absence (Figure 8). All these observations indicate that the main contribution of KlGγ in the *K. lactis* signaling pathway is to enhance the membrane anchoring of KlGβ provided by KlGα.

In *S. cerevisiae*, both Gβ and Gγ are required for activation of downstream elements and inactivation of Gγ prevents association of Gβ with the scaffold protein Ste5p (Whiteway *et al.*, 1995). However our results indicate that in *K. lactis*, KlGβ can activate effector proteins in the absence of KlGγ.

The fact that a heterotrimeric G protein can function as a heterodimer (GαGβ) supports the proposed model of G

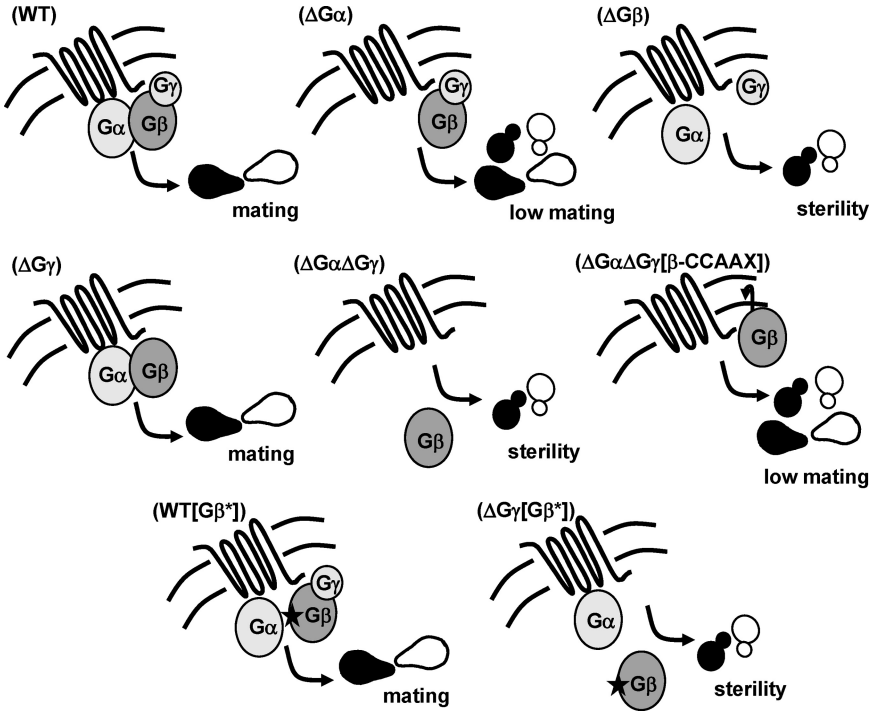

Figure 8. Role of G protein subunits in the *K. lactis* mating pathway. The three subunits of the trimeric protein play positive roles in the transmission of the pheromone stimulus. Although the Gβ subunit is the main transducer, Gα is required at some degree, and Gγ is dispensable. To be active, Gβ has to be tethered to the membrane by action of Gα and/or Gγ. Soluble Gβ is unable to activate the mating system. Gβ fused to a CCAAX motif (curved arrow) partially bypasses lack of Gα and Gγ. Gβ subunit defective in its interaction with Gα (indicated by the black star) can function only in presence of Gγ.

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 Δ*KlG*γ 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 Gα subunits, one Gβ, and one Gγ. Although Gα2 is implicated in cAMP signaling and mating (Bennett and Johnson, 2006), Gα1 and the G$\beta\gamma$ dimer are required for the pheromone response pathway. Loss of either Gα1 or Gβ produces full sterility (Dignard *et al.,* 2008), but effects of Gγ inactivation remain to be investigated. *Cryptococcus neoformans* contains three Gα subunits, one Gβ, and two Gγs. Gα1 regulates cAMP signaling (Alspaugh *et al.,* 1997), whereas Gα2 and Gα3 have opposite roles in response to pheromones. Gα2 activates mating, whereas Gα3 inhibits mating (Hsueh *et al.,* 2007). Inactivation of Gβ or Gγ2 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 Gα2, 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 Gα1 alone and it seems that G$\beta\gamma$ 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 GγS^{87} 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 Δ*KlG*α mutant to form diploids is totally eliminated when KlGγ is inactivated, indicating that in the absence of KlGα, 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 KlG$\beta\gamma$ 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 is a negative regulator of the function of Gα1 in the mating pathway in *S. pombe*, but is not required for signaling (Goddard *et al.,* 2006). It appears that 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 Gpb1p and Gpb2p regulate cAMP signaling by inhibiting Gα2 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 Gα subunit is N-myristoylated and S-palmitoylated, and the Gγ is farnesylated and S-palmitoylated (Dohlman and Thorner, 2001). Although inhibition of either Gα modifications in *S. cerevisiae* results in partial release of G$\beta\gamma$, it has been observed that some G$\beta\gamma$ dimers remain attached to the membrane,

inducing constitutive signaling (Hirschman *et al.*, 1997). On the other hand, inhibition of Gγ farnesylation results in a sterile phenotype, whereas inhibition of Gγ S-palmitoylation significantly reduces its function (Whiteway 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 Å^2 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 GβR^{136}, suggesting that this mutant Gβ has lost complete interaction with Gα (Whiteway *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 GαGβ 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 Gβ 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 Δ*KlGβ*Δ*KlGγ* 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 deficient 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 Δ*KlGγ* 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.

ACKNOWLEDGMENTS

We thank Guadalupe Codiz and Minerva Mora (Molecular Biology Unit, IFC) for technical assistance and the staff of the Computer Unit, IFC. We also thank Miguel Shingu and Marisela Bolaños for technical assistance. This work was partially funded by Grants 44178 from CONACyT, México and IN211906 from PAPIIT, DGAPA, UNAM. R.N. was supported by a PhD fellowship from CONACyT. This study was performed in partial fulfillment of the requirements for the Ph.D. degree in Ciencias Biomédicas of R.N.

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