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Efficient signal transduction by a chimeric yeast–mammalian G protein α subunit Gpa1–Gsα covalently fused to the yeast receptor Ste2

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Saccharomyces cerevisiae **uses G protein-coupled receptors for signal transduction. We show that a fusion protein between the α-factor receptor (Ste2) and the Gα subunit (Gpa1) transduces the signal efficiently in yeast cells devoid of the endogeneous *STE2* and *GPA1* genes. To evaluate the function of different domains of Gα, a chimera between the N-terminal region of yeast Gpa1 and the C-terminal region of rat Gsα has been constructed. This chimeric Gpa1–Gsα is capable of restoring viability to haploid *gpa1*Δ cells, but signal transduction is prevented. This is consistent with evidence showing that the C-terminus of the homologous Gα is required for receptor–G protein recognition. Surprisingly, a fusion protein between Ste2 and Gpa1–Gsα is able to transduce the signal efficiently. It appears, therefore, that the C-terminus of Gα is mainly responsible for bringing the G protein into the close proximity of the receptor's intracellular domains, thus ensuring efficient coupling, rather than having a particular role in transmitting the signal. To confirm this conclusion, we show that two proteins interacting with each other (such as Snf1 and Snf4, or Ras and Raf), each of them fused either to the receptor or to the chimeric Gα, allow efficient signal transduction.**

Keywords: chimeric Gα subunit/Gα C-terminus/Gpa1/Gsα/receptor–Gα fusion protein

Introduction

A major class of signal transducing proteins in eukaryotic cells, from yeasts to mammals, is composed of seven-transmembrane domain receptors coupled to GTP-binding proteins (G proteins). This class includes several hundred members which can respond to a variety of agents, such as hormones, neurotransmitters, odorants and light signals (reviewed by Baldwin, 1994; Strader *et al.*, 1994). Upon ligand binding, the receptors activate αβγ heterotrimeric G proteins, promoting the exchange of bound GDP with GTP on Gα and the subsequent dissociation of Gα from Gβγ. Both the activated, GTP-bound Gα and/or the free Gβγ dimer regulate specific target effectors, stimulating or inhibiting their function. Once GTP is hydrolyzed to GDP, the α and βγ subunits reassociate and the G protein returns to the receptor in its resting state (reviewed by Conklin and Bourne, 1993; Neer, 1995; Hamm and Gilchrist, 1996).

The yeast *Saccharomyces cerevisiae* is a useful organism for studying signal transduction pathways regulated by G protein-coupled receptors. The two haploid cell types (**a** and α) each secrete a peptide pheromone (**a**-factor and α-factor, respectively), that acts on the other cell type to promote conjugation, resulting in the formation of **a**/α diploid cells. The **a** and α mating factors bind to cell surface receptors of the seven-transmembrane domain type (encoded by the *STE3* and *STE2* genes, respectively). In response to pheromone binding to the receptors, the G protein α subunit (Gpa1) replaces bound GDP with GTP and dissociates from the G protein βγ complex, which in turn triggers a cascade of events leading to transcriptional induction of specific genes, stimulation of morphological changes and inhibition of growth (reviewed by Kurjan, 1992, 1993; Sprague and Thorner, 1992).

The deletion of the gene encoding the yeast Gα subunit (*GPA1*) results in lethality in haploid cells, because the free Gβγ complex constitutively activates the pathway which leads to growth inhibition. Mammalian Gα subunits (such as Gsα, Goα or Giα) or chimeric yeast–mammalian Gα subunits are able to bind to the yeast Gβγ complex, and thereby restore viability to haploid cells with the deleted *GPA1* gene (*gpa1*Δ) (Dietzel and Kurjan, 1987; Kang *et al.*, 1990). However, mammalian or even chimeric yeast–mammalian Gα subunits do not allow signal transduction in yeast: presumably the mammalian Gα cannot interact with the yeast receptor. This is consistent with evidence showing that the C-terminus of the homologous Gα is required for receptor–G protein recognition.

To investigate specific interactions between receptors, G proteins and effectors in intact cells, Bertin *et al.* (1994) engineered a receptor–transducer fusion protein, by covalently linking the β_2-adrenergic receptor to the Gsα subunit. They found that this fusion protein was able to restore efficient cellular signaling in mutant animal cells which, although expressing endogenous β_2-adrenergic receptors, were devoid of endogenous Gsα subunits. Very recently, a fusion protein between the α_{2A}-adrenergic receptor and Gi1α was shown to be functional, as judged by the induced stimulation of the G protein's GTPase activity (Wise *et al.*, 1997).

We decided to undertake a similar approach in yeast cells, where it is possible to obtain mutants with deletions of both the *GPA1* gene and the *STE2* gene. We show here that not only is a fusion protein between the Ste2 receptor and the Gpa1 protein functional in signal transduction, but surprisingly, even a fusion protein between the yeast Ste2 receptor and a chimeric yeast–mammalian Gα subunit is able to transduce the signal efficiently. This result is in apparent contrast with the above-mentioned inability of

the chimeric Gα to interact with the yeast receptor. In other words, when the Ste2 receptor and the chimeric yeast–mammalian Gα subunit are separate, there is no response at all to α-factor, but when these two proteins are fused together, there is efficient signal transduction. We explain this finding by suggesting that the C-terminus of the Gα subunit is mainly responsible for bringing the G protein into the close proximity of the receptor's intracellular domains, thus ensuring efficient coupling. This function becomes dispensable when the two proteins are covalently fused.

Results

The Ste2–Gpa1 fusion protein complements the deletion of the endogenous GPA1 gene

A fusion protein between the *S.cerevisiae* α-factor receptor (Ste2) and G protein α subunit (Gpa1) was obtained, as described in Materials and methods, by joining the entire *STE2* gene, except for the bases encoding the last 62 amino acids of the long cytoplasmic tail, to the 5′-end of the complete *GPA1* gene. This construct was inserted into either the multicopy episomal plasmid YEp24 or into the single-copy integrating plasmid YIp5, both of which carry the *URA3*-selectable marker.

Two separate questions could be addressed concerning the functionality of the Ste2–Gpa1 fusion protein: is the fusion protein able to function as a Gα subunit, and is it also able to function as a receptor?

To answer the first question, the recombinant plasmids encoding the fusion protein were transformed into the yeast diploid strain RM7, where both alleles of *STE2* and a single allele of *GPA1* were deleted, and the diploids were sporulated and dissected. In haploid cells, the deletion of the *GPA1* gene would result in constitutive activation of the signal transduction pathway and, therefore, inhibition of growth. Based upon this, we expected the diploid strain RM7 without a Gpa1-encoding plasmid to give rise to two viable spores (those that received the wild-type chromosomal *GPA1* allele) and two spores unable to grow (those that received the *gpa1*Δ mutant allele). However, in the presence of a plasmid encoding a functional Gα subunit, four viable spores could arise, provided that the two *gpa1*Δ spores received the plasmid. Figure 1 shows that the Ste2–Gpa1 fusion protein is able to complement the deletion of *GPA1*, yielding tetrads with more than two viable spores. Because the fusion gene is under the *MAT***a**-specific promoter of *STE2*, only those spores that are *MAT***a** will express the fusion protein and thus be viable. This fact explains why some tetrads do not contain four viable spores, but only three or two. The ability of the Ste2–Gpa1 fusion protein to complement the deletion of *GPA1* is seen when either a multicopy plasmid or a single-copy plasmid, which was integrated at the *STE2* chromosomal locus, are used (Figure 1C and D).

To investigate further the functionality of the Ste2–Gpa1 fusion protein, cells not containing the wild-type Gpa1 protein, which could interfere with the analysis, were needed. Therefore, to discriminate the spores that received the wild-type chromosomal *GPA1* allele from those that inherited the *gpa1*Δ mutation, the cells of the tetrads were examined microscopically. A pattern was observed where two colonies of each tetrad contained

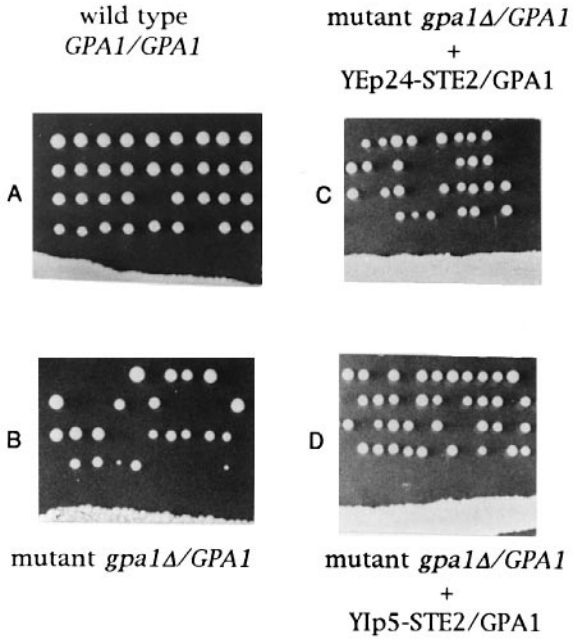


Fig. 1. Complementation of *gpa1*Δ growth defect by the fusion protein Ste2–Gpa1. Yeast diploid strains were sporulated and dissected. Homozygous wild-type *GPA1*/*GPA1* diploid strain GDS94 gives rise to four viable spores (**A**); heterozygous mutant *gpa1*Δ/*GPA1* strain RM7, with the deletion of one *GPA1* allele, gives rise to only two viable spores (**B**); a multicopy plasmid (YEp24) or an integrating plasmid (YIp5) encoding a Ste2–Gpa1 fusion protein complement the *gpa1*Δ mutation, giving rise to three or four viable spores (**C** and **D**).

well-growing cells while the other one or two colonies contained 2–5% morphologically aberrant cells, most likely due to the occasional loss of the complementing plasmid and subsequent activation of the inhibiting pathway. We therefore assigned the wild-type phenotype to the *GPA1* cells, and the aberrant one to the *gpa1*Δ mutant. Confirmation of this line of reasoning was provided by the fact that this pattern was observed only when cells carried the episomal recombinant plasmid, and not when they contained the integrated one, in which case plasmid loss is a rare event. Because no aberrant phenotype was visible with the integrating plasmid, we utilized another method to distinguish between the *gpa1*Δ mutants and the wild-type *GPA1* colonies. We streaked the colonies arising from the tetrads onto plates containing 5-fluoroorotic acid (5-FOA), which counterselect for the presence of the *URA3* marker: rare events of popping-out of the *URA3* integrating plasmid could give rise to Ura$^-$ viable cells only in wild-type *GPA1* segregants, whereas in *gpa1*Δ mutants the presence of the *URA3* plasmid containing the *STE2–GPA1* fusion gene is absolutely necessary for growth, and no pop-out events could be selected for. Indeed, two colonies from each tetrad were growing on 5-FOA plates, while the other one or two were not. The latter were considered to be *gpa1*Δ. Consistent with these observations, when the cells containing the episomal *URA3* plasmid YEp24-STE2/GPA1 were grown in a non-selective liquid medium, those assumed to bear the chromosomal *GPA1* gene allowed the loss of the plasmid and became Ura$^-$, whereas those assumed to be *gpa1*Δ cells did not, indicating that the retention of the plasmid was necessary for growth.

We concluded from this analysis that the Gα subunit

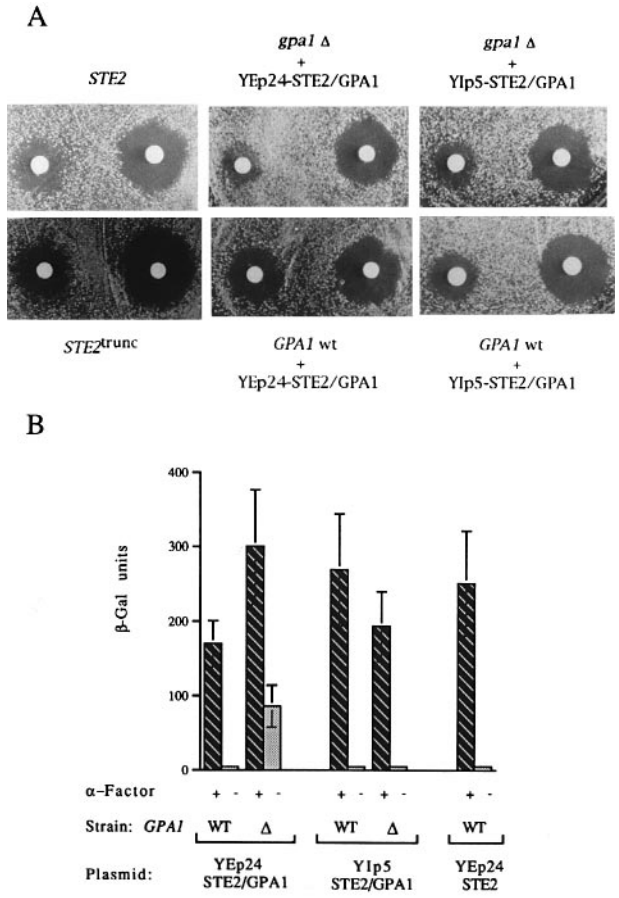


Fig. 2. Response of the Ste2–Gpa1 fusion protein to α-factor. Signal transduction by the Ste2–Gpa1 fusion protein was analyzed by its ability to arrest the cell cycle (A) and to induce gene expression (B) in response to α-factor in *MAT***a** segregants of strain RM7, with (*GPA1* wt) or without (*gpa1*Δ) the chromosomal *GPA1* gene, carrying an episomal, multicopy plasmid (YEp24), or an integrating plasmid (YIp5), encoding the Ste2–Gpa1 fusion protein. The controls were wild-type *GPA1* RM7 segregants expressing the wild-type or the truncated (lacking the last 62 amino acids of the cytoplasmic tail) Ste2 receptor. (**A**) Growth inhibition induced by α-factor was analyzed by the halo assay. Different doses (left, 0.4 μg; right, 4 μg) of α-factor were spotted on filter disks on a lawn of cells. Plates were photographed after 48 h of incubation at 30°C. (**B**) Pheromone-induced expression of *FUS1–lacZ* was checked by the β-galactosidase assay. Cells were incubated with (+) or without (–) α-factor (2.5 μg/ml) for 6 h. The activation of the signal transduction pathway was measured by assaying the β-galactosidase activity in permeabilized cells. The data represent averages of three experiments; error bars indicate 1 SD.

Gpa1, even when fused to the Ste2 receptor, is indeed able to complement efficiently the deletion of the endogenous *GPA1* gene.

The Ste2–Gpa1 fusion protein also complements the deletion of STE2

The presence of three or four viable spores shown in Figure 1 is an indication that the fusion protein Ste2–Gpa1 is able to bind the G protein βγ complex, and thereby keeps the signal transduction pathway in the resting state. However, it does not reveal whether or not the fusion protein is able to transmit the signal upon binding of the α-factor to its receptor moiety.

This question was addressed by several approaches. The halo assay (Figure 2A) is used to measure the inhibition of growth caused by the α-factor binding to the receptor and subsequent onset of signal transduction. It can be seen that cells devoid of the endogenous Ste2 receptor and Gpa1 subunit, but carrying a plasmid encoding the fusion protein Ste2–Gpa1, have a response to the α-factor similar to wild-type cells. The size of the halo is approximately equivalent in cells harboring both types of recombinant plasmids, either multicopy or integrating. Moreover, the presence of the chromosomal wild-type *GPA1* gene, together with the fusion gene, does not increase the efficiency of the response significantly.

The fusion protein lacks the last 62 amino acids of the Ste2 receptor, which are part of the long cytoplasmic tail, a region known to be involved in the desensitization process of the signal transduction pathway. Its loss causes a supersensitive phenotype (Konopka *et al.*, 1988; Reneke *et al.*, 1988), and therefore it could be argued that the efficiency of signal transduction observed with the Ste2–Gpa1 fusion protein is due to its inability to undergo desensitization. To rule out this possibility, we tested the response of a truncated Ste2 receptor, lacking those 62 amino acids, to α-factor in *GPA1* cells. We found no significant increase in the signal transduction by truncated Ste2 as compared with wild-type Ste2 (Figure 2A).

Induction of FUS1–lacZ expression by α-factor binding to the Ste2–Gpa1 fusion protein

A second, more sensitive and quantitative approach to analyze signal transduction in yeast involves assaying the activity of a transfected *Escherichia coli* β-galactosidase gene, placed under the control of the yeast *FUS1* gene promoter. *FUS1* is one of several yeast genes activated by the signal transduction pathway when α-factor binds to the Ste2 receptor. The β-galactosidase activity is therefore a measure of the induction level of the signal transduction pathway.

As shown in Figure 2B, incubating α-factor with haploid *gpa1*Δ cells harboring the fusion protein Ste2–Gpa1 causes a strong induction of *FUS1–lacZ* expression, comparable with expression levels obtained in wild-type cells. Cells that had the chromosomal *GPA1* gene deleted, but carried the fusion gene *STE2–GPA1* on the episomal plasmid YEp24, had a basal level of *FUS1–lacZ* expression higher than cells possessing the wild-type chromosomal *GPA1* gene. This basal activity could be due to an impairment of the interaction between the Gα moiety of the fusion protein and the Gβγ complex. More probably, however, it is caused by the occasional loss of the episomal plasmid encoding the fusion protein in some of the cells of the culture, which would render the Gβγ complex free to activate the pathway. In fact, when the fusion gene was inserted into the integrating plasmid YIp5, there was no substantial basal activity (Figure 2B). Consistently, when a wild-type *GPA1* gene is placed on an episomal plasmid, it produces a basal activation of the signal transduction pathway in *gpa1*Δ cells (data not shown).

A fusion protein between the Ste2 receptor and a chimeric yeast–mammalian Gα subunit is able to transduce the signal efficiently

It has been shown previously that a mammalian G protein α subunit is able to complement the deletion of the *GPA1* gene in *S.cerevisiae* (Dietzel and Kurjan, 1987). The

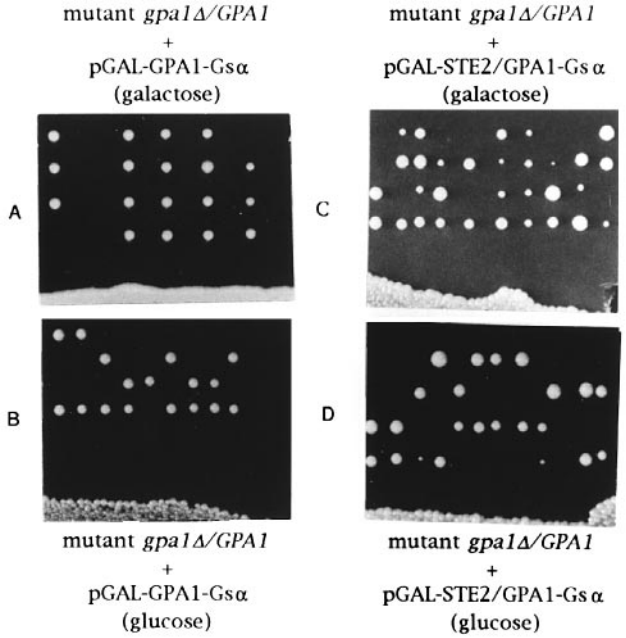


Fig. 3. Complementation of *gpa1*Δ growth defect by a chimeric yeast–mammalian Gα subunit fused to the Ste2 receptor. Yeast diploid strain RM7 (*gpa1*Δ/*GPA1*) was sporulated and dissected. A pGAL plasmid encoding a yeast–mammalian chimeric Gα subunit Gpa1–Gsα, or a fusion protein Ste2–Gpa1–Gsα, complements the *gpa1*Δ mutation, giving rise to four viable spores when cells are grown on a galactose-containing medium (**A** and **C**), and to only two viable spores on a glucose medium (**B** and **D**).

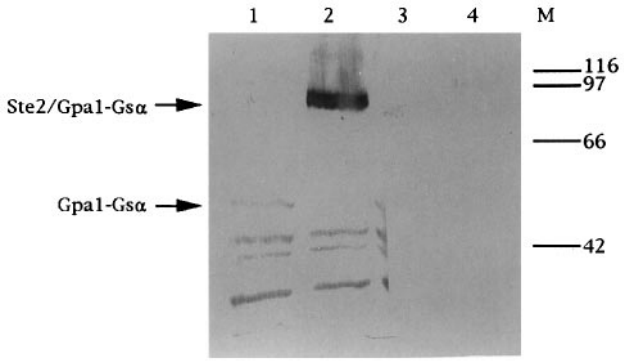


Fig. 4. Visualization of the Ste2–Gpa1–Gsα fusion protein using anti-Gsα antibodies. The immunoblot shows proteins extracted from membranes (lanes 1 and 2) or from cytoplasm (lanes 3 and 4) of *gpa1*Δ segregants of strain RM7, containing the fusion protein between the Ste2 receptor and the Gpa1–Gsα chimera (lanes 2 and 3) or the Gpa1–Gsα chimera alone (lanes 1 and 4). The antibodies were raised against the C-terminus of Gsα. The molecular weights of markers are shown in kDa (M).

efficiency of complementation is increased if a chimera between the N-terminal region of the yeast Gα subunit and the C-terminal region of a mammalian Gα is utilized (Kang *et al.*, 1990). However, the complementation by mammalian Gα or by the yeast–mammalian chimeric Gα is only limited. These Gα subunits are able to interact with the yeast Gβγ complex, but not with the yeast pheromone receptor: therefore, a yeast *gpa1*Δ strain containing either the mammalian or the yeast–mammalian chimeric Gα subunit is viable but sterile, and does not respond to pheromones (Kang *et al.*, 1990). This is consistent with the generally accepted model according to which the C-terminus of Gα is involved in receptor recognition (see Discussion). We wondered, therefore, whether a fusion protein between Ste2 and a chimeric yeast–mammalian Gα subunit would be active in signal transduction.

First, we constructed a chimera between yeast Gpa1 and rat Gsα, as described in Materials and methods. It contains the N-terminal 362 amino acids of Gpa1 and the C-terminal 128 amino acids of rat Gsα. The junction site is within a highly conserved sequence, and therefore it is likely that this chimera would retain the G protein's normal structure. The *GPA1–Gsα* chimeric gene was placed under the control of the *GAL1* promoter into the very high copy number plasmid pGAL (derived from pEMBLyex2), which would compensate the possible low efficiency of the chimeric Gα in sequestering the Gβγ complex. This plasmid was introduced into the *GPA1*/*gpa1*Δ diploid strain RM7, and the diploids were sporulated and dissected. Figure 3A shows that the chimera Gpa1–Gsα is able to complement the deletion of the *GPA1* gene efficiently, producing tetrads with four viable spores. As expected, when the tetrads were grown on glucose instead of galactose, only two viable spores arose (Figure 3B).

We then constructed a fusion protein between the Ste2 receptor and the yeast–mammalian chimera Gpa1–Gsα, as described in Materials and methods. Immunoblot analysis verified the proper presence of the fusion protein Ste2–Gpa1–Gsα with the expected molecular weight (Figure 4). This fusion protein was found to complement the lethality of the *GPA1* deletion in haploid cells, as shown by dissection of tetrads of the *GPA1*/*gpa1*Δ diploid strain RM7 (Figure 3C).

Surprisingly, when we checked the ability of *gpa1*Δ RM7 segregants containing the fusion protein Ste2–Gpa1–Gsα to respond to α-factor, a strong response was obtained, both in the halo assay (Figure 5A) and in the *FUS1–lacZ* induction assay (Figure 5B). On the other hand, as expected, *gpa1*Δ RM7 segregants expressing, separately, the chimera Gpa1–Gsα and the Ste2 receptor were found to be completely defective in α-factor response, in the halo assay (Figure 5A) as well as in the β-gal assay (Figure 5B). The elevated basal activity in *gpa1*Δ cells could be caused by the occasional loss of the plasmid encoding the chimeric Gα (even though this is a very high copy number plasmid), but it is also likely that the Gpa1–Gsα chimera, as opposed to wild-type Gpa1, is relatively inefficient in sequestering the Gβγ complex, thereby causing a certain amount of activation of the pathway.

To rule out the possibility that gene conversion or other recombination processes occurred during meiotic division between the chromosomal *GPA1* gene and the chimeric *GPA1–Gsα* gene carried by the plasmid, we recovered the plasmid from several *gpa1*Δ RM7 segregants, and checked its identity. We could verify, by restriction mapping and sequencing of a dozen independently rescued plasmids, that these indeed contained the chimeric gene.

We conclude, therefore, that when the Ste2 receptor (both the wild-type and the 62 amino acid-less truncated form) and the chimeric yeast–mammalian Gα subunit are separate, there is no response at all to α-factor. On the

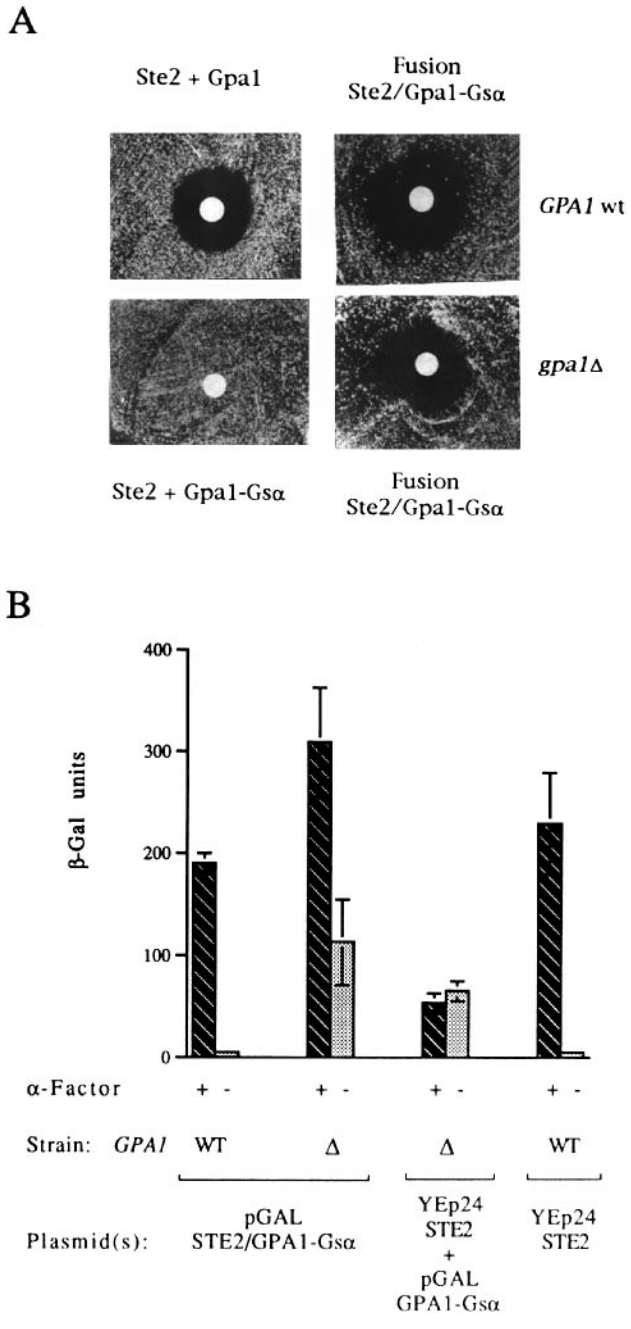
other hand, when the two proteins are covalently fused together, there is a very efficient signal transduction.

Ste2/Snf1 couples to Gpa1–Gsα/Snf4 and Ste2/Raf couples to Gpa1–Gsα/Ras to transmit the signal

The results obtained with the Ste2–Gpa1–Gsα fusion protein suggest that the specific interaction of the receptor with the C-terminus of Gα is mainly necessary to bring the two proteins into close proximity, rather than having a particular role in transmitting the signal. The fusion between the receptor and the Gα subunit overcomes the requirement for this specific interaction. If this hypothesis is correct, we reasoned that it should be possible to reach the same goal by utilizing two other proteins (say X and Y), interacting with each other, each of them fused to a separate component of the signal transduction system, either the receptor or the Gα subunit. It will be the interaction of the two separate X and Y proteins which will bring the receptor and Gα into close proximity, thus ensuring the transmission of the signal between the latter two proteins (Figure 6).

To test this hypothesis, we constructed two pairs of interacting hybrid proteins: Ste2/Snf1 matching with Gpa1–Gsα/Snf4, and Ste2/Raf matching with Gpa1–Gsα/Ras. Snf1 and Snf4 are required for glucose derepression in *S.cerevisiae* (Johnston and Carlson, 1992) and previously were shown to interact in the two-hybrid system (Fields and Song, 1989) and also by other means (Celenza *et al.*, 1989). Ras and Raf are oncoproteins which also were shown to interact with each other (Vojtek *et al.*, 1993; Van Aelst *et al.*, 1993). We have used the constitutively active form of Ras (valine at position 12), which presumably would interact with Ste2/Raf more efficiently. According to our hypothesis, the interaction between Snf1 and Snf4, or between Raf and Ras, should bring the Ste2 receptor and the Gpa1–Gsα protein (which by themselves are unable to interact, as we have shown above) close enough to each other so that they could couple and transmit the signal. In Figure 7 we show that this is indeed the case. Haploid yeast cells responded to α-factor only when both matching hybrid proteins (Ste2/Snf1 with Gpa1–Gsα/Snf4, or Ste2/Raf with Gpa1–Gsα/Ras) were present (Figure 7A and B, respectively). The level of *FUS1–lacZ* induction in these cells was considerably higher than the basal activity without α-factor or the level obtained in the controls containing only one hybrid protein.

To ascertain the specificity of the interaction further, we show that non-matching hybrid proteins (i.e. Ste2/Snf1 with Gpa1–Gsα/Ras, and Ste2/Raf with Gpa1–Gsα/Snf4) are unable to transduce the signal (Figure 7B).



Discussion

The main finding of this study is that two proteins (in this case, a seven-transmembrane domain receptor and a G protein α subunit), which are unable to interact with each other because one of them bears a heterologous portion (the C-terminal region of the mammalian Gα subunit replacing the corresponding region in the yeast Gα), nevertheless can be functionally coupled if they are

Fig. 5. Efficient signal transduction by a chimeric yeast–mammalian Gα subunit covalently fused to the Ste2 receptor, analyzed by its ability to arrest the cell cycle and to induce gene expression in response to α-factor. (**A**) *MAT***a** segregants of strain RM7, with (*GPA1* wt) or without (*gpa1*Δ) the chromosomal *GPA1* gene, expressing the Ste2–Gpa1–Gsα fusion protein, were analyzed for growth inhibition by the halo assay. The controls were wild-type *GPA1* RM7 segregants expressing the wild-type Ste2 receptor, and *gpa1*Δ RM7 segregants expressing, separately, the Ste2 receptor and the yeast–mammalian chimera Gpa1–Gsα. Synthetic α-factor (4 μg) was spotted on filter disks on a lawn of cells. Plates were photographed after 48 h of incubation at 30°C. (**B**) Pheromone-induced expression of *FUS1–lacZ* was checked by the β-galactosidase assay. *MAT***a** segregants of strain RM7, with (*GPA1* wt) or without (*gpa1*Δ) the chromosomal *GPA1* gene, carrying a pGAL plasmid encoding the Ste2–Gpa1–Gsα fusion protein or carrying two separate plasmids encoding the Ste2 receptor and the chimeric Gpa1–Gsα, respectively, were incubated with (+) or without (–) α-factor (2.5 μg/ml) for 6 h. The control was a wild-type *GPA1* strain carrying a YEp24-STE2 plasmid. The activation of the signal transduction pathway was measured by assaying the β-galactosidase activity in permeabilized cells. The data represent averages of three experiments; error bars indicate 1 SD.

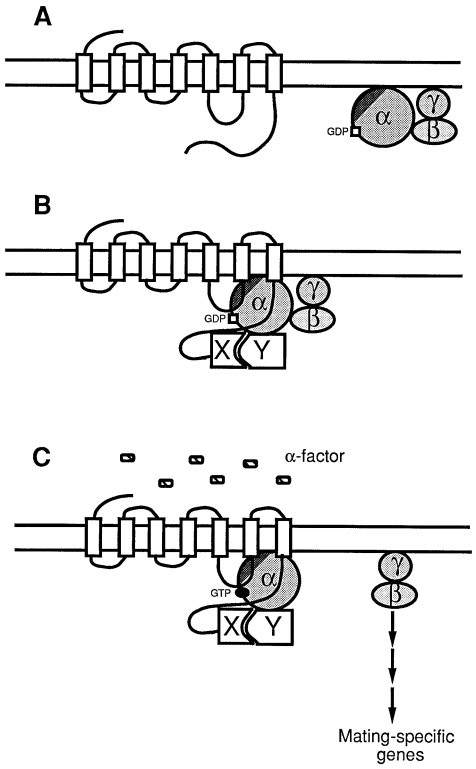


Fig. 6. Coupling of the receptor to the Gα subunit through the interaction of two sticky proteins. (**A**) A seven-transmembrane domain receptor is unable to interact with a G protein α subunit bearing a heterologous C-terminus (darkened segment). (**B**) Two hybrid proteins are constructed: one between the receptor and protein X, and another between Gα and protein Y. The interaction between the two proteins X and Y allows coupling of the Gα subunit to the receptor (the regions of contact between the receptor and Gα are only indicative). (**C**) The presence of the ligand activates the receptor which triggers the GDP/GTP exchange on the Gα subunit and the release of the βγ complex. The latter in turn activates a cascade of reactions, leading to the induction of specific genes.

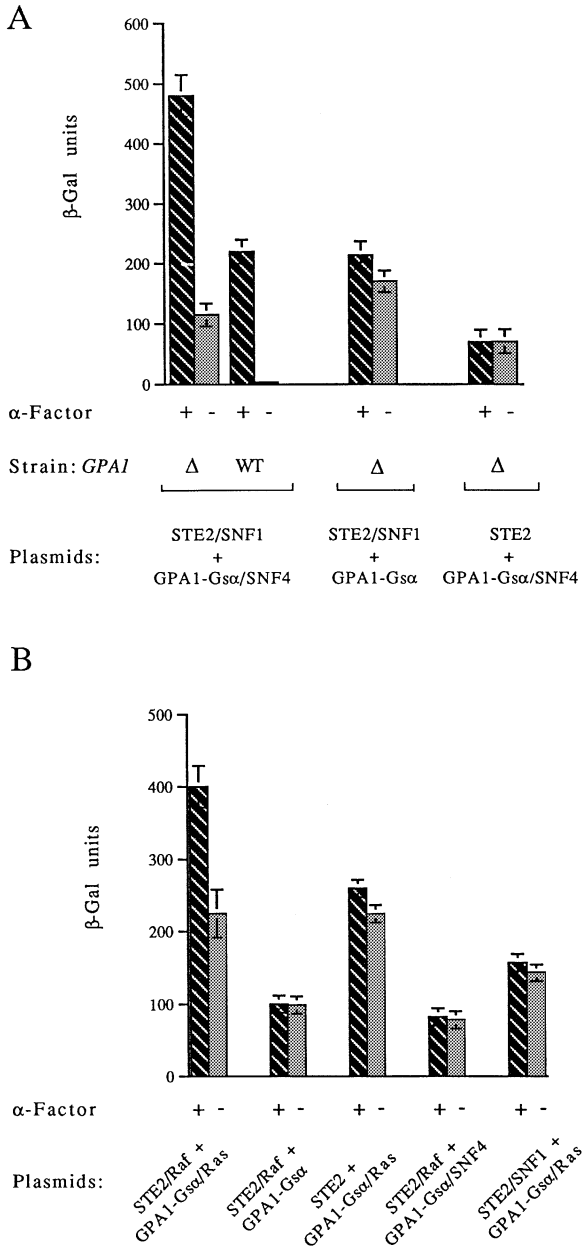


Fig. 7. The interactions between Snf1 and Snf4 and between Raf and Ras allow receptor–Gα coupling. (**A**) *MAT***a** segregants of strain RM7, with (*GPA1* wt) or without (*gpa1*Δ) the chromosomal *GPA1* gene, carrying the plasmids YIp5-STE2/SNF1 and pGAL-GPA1-Gsα/SNF4, were incubated with (+) or without (–) α-factor (2.5 μg/ml). Controls are cells expressing only one hybrid protein, i.e. cells carrying the plasmids YIp5-STE2/SNF1 and pGAL-GPA1-Gsα, or YEp24-STE2 and pGAL-GPA1-Gsα/SNF4. (**B**) *MAT***a**, *gpa1*Δ strain RM20 (devoid of the plasmid pYX212-GPA1), carrying the plasmids pYX123-STE2/Raf and pYX242-GPA1-Gsα/Ras, were incubated with (+) or without (–) α-factor (2.5 μg/ml). Controls are cells expressing only one hybrid protein, i.e. cells carrying the plasmids pYX123-STE2/Raf and pYX242-GPA1-Gsα, or pYX123-STE2 and pYX242-GPA1-Gsα/Ras. Other controls are cells expressing non-matching hybrid proteins, i.e. cells carrying the plasmids pYX123-STE2/Raf and pGAL-GPA1-Gsα/SNF4, or pYX123-STE2/SNF1 and pYX242-GPA1-Gsα/Ras. The activation of the signal transduction pathway was measured by assaying the β-galactosidase activity in permeabilized cells. The data represent averages of three experiments; error bars indicate 1 SD.

covalently linked together. In other words, when the yeast α-factor receptor Ste2 and the chimeric yeast–mammalian Gα subunit Gpa1–Gsα are separate, there is no response at all to α-factor, but when the two proteins are fused together, there is efficient signal transduction.

In the case of the yeast G protein α subunit, our results suggest that the C-terminal region is mainly responsible for bringing the Gα subunit into close contact with the Ste2 receptor, thus ensuring efficient coupling during signal transduction. The activation of Gα by the ligand-bound receptor leading to GDP release and GTP binding, however, would occur through a different domain of Gα and, presumably, also involve a different region of the receptor. The substitution of the C-terminal region of the yeast Gα with a heterologous one abolishes the specific interaction required to ensure efficient recognition between the receptor and Gα, rendering the chimeric Gα unable to couple to the receptor. However, when the receptor and the chimeric Gα are covalently linked, it appears that the specific interaction between the C-terminal region of Gα and the receptor is no longer required for signal transduction to proceed efficiently.

The importance of the C-terminal region of Gα subunits in the interaction with seven-transmembrane domain receptors is well documented (reviewed by Conklin and Bourne, 1993; Neer, 1995; Hamm and Gilchrist, 1996). Mutations in this region impair Gα's function (Sullivan

et al., 1987; Hirsch *et al.*, 1991; Garcia *et al.*, 1995; Kallal and Kurjan, 1997) or alter its specificity (Conklin *et al.*, 1993). Moreover, peptide analogs of the C-terminus of Gα have been shown to mimic its function (Rasenick *et al.*, 1994; Martin *et al.*, 1996).

Besides the C-terminus, other regions of Gα have been implicated in the interaction with the receptor (Cerione *et al.*, 1986; Kleuss *et al.*, 1991; Wall *et al.*, 1995). By constructing chimeric Gα subunits, it has been shown that multiple regions contribute to the specificity of interaction with the receptor (Lee *et al.*, 1995). More recently, Bourne and collaborators have undertaken theoretical (Lichtarge *et al.*, 1996) and experimental (Onrust *et al.*, 1997) studies aimed at identifying functional surfaces on Gα (through which Gα is triggered to exchange GDP to GTP in response to receptor activation) interacting with the receptor and Gβγ subunits. They have identified two clusters of Gα residues implicated in these interactions. Following the nomenclature of Lambright *et al.* (1994, 1996), one surface includes part of helix α5, strand β6, the α4–β6 loop and the C-terminal tail. These elements are considered to be part of the surface interacting with the receptor. The second cluster comprises residues of the N-terminus, helix α2 (corresponding to the 'switch 2' region), and strands β1–β3. Part of this surface directly contacts Gβγ in crystals of the heterotrimeric G protein (Lambright *et al.*, 1996). According to the proposed model (Lichtarge *et al.*, 1996; Onrust *et al.*, 1997), the activated receptor cooperates with Gβγ to open the nucleotide-binding pocket of Gα by an 'action at a distance' mechanism. The main contributing elements would be the α5 helix, located in the C-terminal region, and the β1 strand, in the N-terminal part, which would jointly sense the receptor and trigger the release of GDP.

Our results are in perfect agreement with this model. The elements of the first cluster of the Gα surface which, according to the model, recognize the receptor are located in the mammalian portion of our chimeric Gα. On the other hand, the residues of the second cluster are included in the yeast segment of the chimeric Gα. The inability of the chimeric Gα to transduce the signal is clearly due to the absence of recognition between the chimeric yeast–mammalian Gα and the yeast receptor. We propose that, at least in *S.cerevisiae*, the C-terminal region of Gα recognizes the receptor while the other cluster of residues, possibly through an interaction with Gβγ, responds to the signal from the activated receptor. The specific recognition of the receptor by the C-terminal region of Gα is no longer required when the chimeric Gα is covalently linked to the receptor. Since in this case the two proteins have been brought close to each other, the transmission of the signal can now proceed through the other site of interaction. This does not mean that the C-terminal region of Gα is completely dispensable when Gα is covalently linked to the receptor, but only that it does not need to be specific. In fact, the C-terminal region is also important to ensure the proper folding of Gα, for example to correctly position the β6–α5 loop (which contacts the nucleotide and is conserved among all the Gα subunits); for this purpose, however, a heterologous C-terminal region works almost as well as the homologous one. The heterologous domain turns out to be defective only in the recognition of the receptor.

An alternative interpretation of our results, which also fits the model of Bourne's group, is that both sites of interaction between the receptor and Gα are necessary for reciprocal binding as well as for transmission of the signal. If one of them is missing, as in the case of the chimeric Gα, the binding force between the two proteins would be too weak to ensure efficient signal transduction. The fusion between the receptor and the chimeric Gα overcomes the necessity for two interaction sites, and just one of them will suffice for efficient coupling.

Signal transduction observed during forced coupling between the yeast receptor and the yeast–mammalian Gα chimera is consistent with results obtained in reconstituted *in vitro* systems. Indeed, at high enough receptor concentrations, a certain degree of selectivity is often lost (reviewed by Dohlman *et al.*, 1991).

If the covalent linkage between the receptor and the chimeric Gα subunit enables them to be in physical proximity and therefore functionally coupled, it should be possible to attain the same goal by using a bridge composed of two other proteins, say X and Y, interacting with each other, each of them fused either to the receptor or to the chimeric Gα. The interaction between X and Y should bring the receptor and the chimeric Gα close enough together to ensure coupling and transmission of the signal (Figure 6).

As we show here, hybrid proteins such as Ste2/Snf1 and Ste2/Raf are able to couple specifically to Gpa1–Gsα/Snf4 and Gpa1–Gsα/Ras, respectively, thereby activating the signal transduction pathway upon α-factor binding (Figure 7).

This finding could be exploited to develop a novel genetic method for detecting protein–protein interactions. The underlying logic is similar to that of the two-hybrid system (Fields and Song, 1989; Bartel and Fields, 1995), in which two interacting proteins are fused, respectively, to a DNA-binding domain and to a transcription-activation domain, thereby stimulating the expression of a reporter gene. The first and still commonly used protein in the two-hybrid system is the Gal4 protein, a very potent activator of transcription in yeast cells when these are grown on a galactose medium. Gal4 is composed of two domains which, if separated, do not allow transcriptional activation unless they are brought into close proximity through the interaction of two other proteins, X and Y. The protein analogous to Gal4 in our case is the fusion protein Ste2–Gpa1–Gsα which, if separated into its two components, the Ste2 receptor and the Gpa1–Gsα chimeric Gα subunit, does not allow signal transduction to proceed, but works efficiently when they are linked together, either covalently or through the interaction of two other proteins attached to them.

One important difference between the two-hybrid system and a system based on G protein-coupled receptors will be that in the former the protein–protein interaction takes place in the nucleus, whereas in the latter it occurs in the cytoplasm or, more precisely, on the cytoplasmic side of the cell membrane.

A different transduction pathway, that involves the activation of Ras by the Sos factor, has been adapted recently to detect protein–protein interactions in the cytoplasm (Aronheim *et al.*, 1997). In this case, the system is based on the finding that targeting of Sos to the plasma

membrane in the vicinity of Ras appears to be the primary mechanism leading to activation of the Ras pathway (Aronheim *et al.*, 1994).

Materials and methods

Media and genetic techniques

Growth media, sporulation, mating and tetrad analysis were as reported in Di Segni *et al.* (1993). Yeast transformation was performed according to Klebe *et al.* (1983). Plasmids were rescued from yeast as described by Robzyk and Kassir (1992).

Strains and plasmids

The following *E.coli* strains were used: DH5α, CC18 and JM110. The *S.cerevisiae* strain GDS94 is a wild-type *GPA1* and *STE2* diploid strain (*MAT***a**/*MAT*α [*ade2-1 his3-11,5 leu2-3,112 trp1-1 ura3-1 can1-100*]). Strain RM7 is a *gpa1*Δ and *ste2*Δ diploid strain (*MAT***a**/*MAT*α *ste2*Δ/*ste2*Δ *GPA1*/*gpa1*Δ *fus1::FUS1-lacZ-[TRP1]/FUS1 ura3-1/ura3-52 lys2/LYS2* [*trp1 ade2-1 his3–11,5 leu2-3,112*]. Strain RM20 is a *gpa1*Δ *ste2*Δ haploid strain (*MAT***a** *gpa1*Δ *ste2*Δ *fus1::FUS1-lacZ*-[*TRP1*] *ura3-52 trp1 ade2-1 his3-11,5 leu2-3,112*) containing the plasmid pYX212 (2μ-*URA3-TPI* promoter; R&D System) carrying the *GPA1* gene. The *STE2* and *GPA1* genes were deleted using the hit-and-run system of Roca *et al.* (1992): for *ste2*Δ, the 1 kb *Pvu*II fragment of the *STE2* coding region was deleted; for *gpa1*Δ, the 0.84 kb *Hin*dIII–*Sph*I fragment of the *GPA1* coding region was deleted. The integration of *FUS1–lacZ* was made using a construct derived from plasmid pSL555 (kindly provided by G.Sprague; McCaffrey *et al.*, 1987).

Plasmid pJBK-036 is a YEp24 vector, a multicopy plasmid (2μ-*URA3*, New England Biolabs) containing the 4.3 kb *Bam*HI *STE2* fragment, kindly provided by L.Hartwell (Konopka *et al.*, 1988). Plasmid YIp5-GPA1 was derived by subcloning the 1.9 kb *Eco*RI fragment containing the *GPA1* gene from plasmid YCp50-SCG1 (kindly provided by J.Kurjan; Dietzel and Kurjan, 1987) into the *Eco*RI site of the integrating plasmid YIp5 (*INT-URA3*, New England Biolabs). Plasmids pYX123 (*CEN-HIS3-GAL*) and pYX242 (2μ-*LEU2-TPI*) are from R&D System. pEMBLyex2 is a 2μ-*URA3-leu2-d-GAL* plasmid (Baldari *et al.*, 1987). pGAL is a very high copy number plasmid derived from pEMBLyex2, carrying only the *leu2-d* marker; pRMU is also derived from pEMBLyex2 and carries only the *URA3* marker.

Construction of chimeric genes

The fusion between the *STE2* gene and the *GPA1* gene was created by ligating a 3.2 kb *Bam*HI–*Pst*I fragment (derived from plasmid pJBK-036), which includes the 5′-flanking region and the coding region of *STE2* (from codon 1 to 369; the last 62 codons of *STE2* are therefore lacking), with the 2.4 kb *Bsm*BI–*Sal*I fragment (from plasmid YIp5-GPA1), which includes the *GPA1* gene from codon 10 to the end and its 3′-flanking region. The following two oligonucleotides were used to allow the ligation between the *STE2* and the *GPA1* fragments and to reconstitute the first nine codons of *GPA1*: 5′-AGCTTTATGGGG-TGTACAGTGAGCACGCAAAC-3′ and 3′-ACGTTCGAAATAC-CCCACATGTCACTCGTGCGTTTGTTAT-5′. The two gene fragments and the oligonucleotides were inserted into plasmids YEp24 or YIp5. For integration in yeast, YIp5-STE2/GPA1 was digested with *Bam*HI.

STE2^{trunc} was obtained by cutting the *STE2* gene at the *Pst*I site, and ligating it to a pair of oligonucleotides containing a stop codon. The truncated *STE2* gene was inserted into the polylinker of pRMU.

The chimeric yeast–mammalian Gα gene *GPA1–Gs*α was created by joining the *Nsi*I site (corresponding to codon 362) of the *GPA1* gene to the *Pst*I site (corresponding to codon 267) of the rat Gsα subunit gene, obtained from pGEM2-Gs (Jones and Reed, 1987). The resultant chimeric Gα protein therefore contains the N-terminal 362 amino acids from yeast Gpa1 and the C-terminal 128 amino acids from rat Gsα. The chimeric *GPA1–Gs*α gene was inserted downstream of the *GAL* promoter into pGAL.

The fusion gene *STE2–GPA1-Gs*α was made by ligating the 3.2 kb *Bam*HI–*Bsm*BI fragment from YEp24-STE2/GPA1 with the *Bsm*BI–*Bam*HI fragment from pGAL-GPA1-Gsα, and inserted into the *Bam*HI site of pGAL.

Plasmid YIp5-STE2/SNF1 was constructed by joining the *Pst*I site of *STE2* to an *Eco*RI site just before the Met codon of *SNF1* (derived from pBTG9-SNF1; Bartel and Fields, 1995) using the following oligonucleotides as adaptors: 5′-GATGATATAGAGAAAG-3′ and 3′-ACGTCTACTATATCTCTTTCTTAA-5′. This chimera was integrated at the *SNF1* locus of yeast by cutting at the single *Bgl*II site of *SNF1*. The chimera *GPA1–Gs*α*/SNF4* was constructed by joining the *GPA1-Gs*α gene, cut at the *Sph*I site (located at eight codons before the end), to the first *Cla*I site of *SNF4* (codon 21), using the following two oligonucletides as an adaptor, which also reconstitute the last eight codons of *GPA1*: 5′-CATCTTCGCCAATACGAGCTGCTCGAAT-3′ and 3′-GTACGTAGA-AGCGGTTATGCTCGACGAGCTTAGC-5′. This chimera was inserted into the pGAL plasmid, downstream of the GAL promoter.

The fusion *STE2/RAF* was constructed by joining the *Pst*I site of *STE2* to an *Eco*RI site preceding the Met codon of c-*RAF* (derived from a pGAD vector; Bartel and Fields, 1995) with the following oligonucleotides as adaptors: 5′-GATGATATAGAGAAAGAATTCGCT-CGCGAG-3′ and 3′-ACGTCTACTATATCTCTTTCTTAAGCGAGCG-CTCTTAA-5′. This fusion was inserted into the plasmid pYX123. The chimera *GPA1–Gs*α*/RAS* was constructed by joining *GPA1–Gs*α cut with *Sph*I to the *RAS* gene. The constitutively active form of Ras, with valine at position 12, was used (derived from pBTM116-RAS; Bartel and Fields, 1995), with the following oligonucleotides as adaptors: 5′-CATCTTCGCCAATACGAGCTGCTCGAATCGTACGTAGG-3′ and 3′-GTACGTAGAAGCGGTTATGCTCGACGAGCTTAGCATGCATCCC-TAG-5′. This chimera was inserted into the plasmid pYX242.

The constructs were checked by sequencing using the dideoxy termination method (T7 DNA polymerase, Pharmacia).

Halo assay

The response to α-factor of *MAT***a** cells was tested by the growth inhibition assay (halo assay). After growth to mid-log phase (A_{600} = 0.2), ~4×10^4 cells were plated on solid medium and different quantities (4 μl of 100 μg/ml to 1 mg/ml) of synthetic α-factor (Sigma) were spotted on sterile filter disks placed on the lawn of cells. The plates were incubated for 1–2 days at 30°C prior to photographing.

β-Galactosidase assay

Cells from a mid-log phase culture (A_{600} = 0.2) were incubated with α-factor at 2.5 μg/ml for 6 h at 30°C. The cells were then spun, resuspended in 1 ml of Z buffer (Miller, 1972) and permeabilized with two drops of chloroform. After 15 min at 30°C, 0.2 ml of 4 mg/ml CPRG (chlorophenol red-β-D-galactopyranoside, Boehringer) were added. Absorbance was read at 574 nm. β-Gal units were calculated using the equation: $U = 1000\times(A_{574})/(\text{time})\times(\text{vol})\times(A_{600})$.

Western blotting

Yeast cells were grown in 50 ml of selective medium and harvested at A_{600} = 0.6. The pellet was resuspended in 1 ml of TEG buffer per gram of cells [TEG is 50 mM Tris pH 7.4, 1 mM EDTA, 10% glycerol, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml pepstatin and 1% β-mercaptoethanol). Cells were disrupted with glass beads (1/3 of volume) at 4°C by five cycles of vortexing for 1 min and incubation on ice for 1 min. After spinning for 10 s, the supernatant was ultracentrifuged at 85 000 *g* for 30 min at 4°C. The pellet was resuspended in a buffer containing 5% SDS, 8 M urea, 1% β-mercaptoethanol and 10% of tracking dye. The samples were incubated at 37°C for 5 min and spun for 5 s; 10 μl aliquots of the supernatant were loaded onto an SDS–10% polyacrylamide gel, and electrophoresed at 200 V for 1 h. The gel was electroblotted to nitrocellulose (Schleicher & Schüll) for 1 h at 150 mA, using the Bio-Rad Minigel system. After staining with Ponceau S (3 min), the filter was washed three times with H_2O, quenched with TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 5 min, and for 30 min with TBST containing 5% bovine serum albumin (BSA). The filter was then incubated for 1 h in TBST containing 1% BSA and antibody anti-Gsα (NEN Dupont; diluted 1:3000–1:5000), and washed three times for 10 min with TBST. Anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega; diluted 1:10 000) was added for 30 min in TBST containing 1% BSA, and the filter was washed three times for 10 min with TBST and once with TBST without Tween. The filter was developed using the reagents (NBT and BCIP) provided by Promega.

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