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# STE2/SCG1-dependent inhibition of STE4-induced growth arrest by mutant STE4$^{\Delta C6}$ in the yeast pheromone response pathway

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**Abstract The yeast pheromone response pathway involves the activation of a heterotrimeric G protein composed by SCG1 ($\alpha$) (also GPA1), STE4 ($\beta$), and STE18 ($\gamma$) subunits by the pheromone-activated receptors STE2 and STE3 in a and $\alpha$ cells, respectively. Upon exchange of bound GDP for GTP in the SCG1 subunit, the release of STE4/STE18 dimer occurs which, in turn causes activation of downstream effectors leading growth arrest and mating competence. Over-expression of STE4 also leads to growth arrest in a STE18 dependent manner. Removal of 6 amino acids from the C-terminus of STE4 rendered a subunit incapable of downstream signalling but still able to interact with STE18. This ΔC6 mutant acts as a dominant negative because it blocks the growth arresting effect obtained by over-expression of STE4. The inhibitory effect of STE4$^{\Delta C6}$ is dependent on the presence of the SCG1 subunit in a *STE2* but not *ste2* background. Inhibition of the growth arresting effect of STE4 by the ΔC6 mutant is not due to competition at the effector site, but rather involves an intrinsic activity of STE2 that is dependent on SCG1.**

*Key words:* STE4; STE18; GPA1; Pheromone receptor; G protein; Signal transduction; Yeast; Structure–Function

## 1. Introduction

Analysis of the epistatic relationships among the genes responsible for initiating the pheromone mating response of *Saccharomyces cerevisiae* has led to the generally accepted view that upon interaction of pheromones with their respective receptors, STE2 for α-pheromone on Mata cells and STE3 for **a** pheromone on Matα cells, this leads to the activation of a heterotrimeric G protein formed of the SCG1 (also GPA1 and CDC70) α subunit, STE4 β subunit and STE18 γ subunit, followed by a cascade of events that leads to transcriptional activation(s) of genes, morphological changes often referred to 'shmoo' formation, growth arrest by destabilization of the three G1 cyclins, CLN1, CLN2 and CLN3, and mating competence [1]. The most proximal element known in this pathway is the recently identified STE20 kinase [2]. Acting presumably through the *STE5* product (of unknown function), it appears to activate a phosphorylation cascade that involves at a minimum the STE11, STE7 and FUS3 kinases, and, downstream of STE7, the transcription factor STE12 responsible for synthesis of the FAR1 product [3]. Of these, FUS3 and FAR1 mediate destabilization of the CLN3 and CLN2 cyclins, respectively [4,5]. The pathway through which CLN1 activity is reduced in response to pheromone activation of the *S. cereviciae* G protein is complex and appears to involve both FUS3 and KSS1 [6] and/or a step 'X' as postulated by Chang and Herskowitz [5]. More recently, the SIG1 protein has been identified as a negative regulator of the pheromone signalling either at the level of the G protein or the STE20 kinase [7].

The roles of the G protein subunits in mediating the pheromone response were studied by Matsumoto and colleagues. They determined that *scg1* cells exhibit constitutive growth arrest and are able to mate [8], that the *scg1* phenotype is suppressed by an *ste4* mutation [8], and that over-expression of STE4 in receptor-less *Δste2 STE18*, but not in *Δste2 ste18* cells also leads to growth arrest [9]. These as well as several other findings [10–12] have led to the concept that growth arrest and the mating response are mediated by the STE4/STE18 complex and independent of the G protein α subunit, SCG1.

In addition to its ability to suppress the growth arrest promoting activity of STE4/STE18, and to be essential in the transmission of the effect of receptor occupancy into STE4/STE18 'activation', SCG1 has been specifically implicated in the mediation of an adaptation response to pheromone. This effects is postulated to come about through direct or indirect interaction with the *SGV1* gene product, a kinase that is structurally related to CDC28-type of kinases [13]. SVG1 may act by down-regulating STE4 and thus release the destabilization of the CLN2 and CLN3 cyclins and/or by stabilizing these cyclins independently of an effect on STE4. In support of a mediating action in the adaptation response to pheromone, an activating mutation of SCG1, Gly$^{50}$ to Val$^{50}$, promotes in a dominant fashion a rapid recovery from pheromone-induced growth arrest. An involvement as a positive signal in mediation of the mating response to pheromone has not been found.

We observed that the C-terminus of the STE4 product is essential for downstream signalling. Thus, while overexpression of full-length STE4 in *ste4* cells led to growth arrest and *shmoo* formation, that of a mutant lacking its six C-terminal amino acids (ΔC6) was ineffective [14]. This was not due to failure to interact with the STE18 subunit. Here we shown that the ΔC6 mutant was not totally inactive, however, for it blocked the growth arresting effect obtained by STE4 in these cells.

## 2. Materials and methods

### 2.1. Strains and media

The yeast strain used in this study were: W303-1A (*Mat* **a**, *ade2, his3, leu2, trp1, ura3, can1-100*), W303-3A (*Mat* **a**, *ade2, his3, leu2, trp1, ura3, can1-100, ste2::LEU2*) and W303-3B (*Mat* α, *ade2, his3, leu2, trp1, can1-100, scg1::URA3* [pGALGsα]) were obtained from J. Kurjan; W303-5A (*Mat* **a**, *ade2, his3, leu2, trp1, can1-100, ste4::URA3*) and W303-1D (*Mat* **a**, *ade2, his3, leu2, trp1, can1-100, ste4::LEU2*) were

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constructed by gene disruption of the *STE4* locus in the W303-1A isogenic strain. The NM522 *E. coli* strain was used to propagate yeast/*E. coli* shuttle vectors and to prepare plasmid DNA for sequencing. SD minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SGal medium contained 0.67% yeast nitrogen base and 2% galactose. SD and SGal minimal media were supplemented with the required amino acids (50 μg/ml) to select for plasmids. 100 μM $Cu^{2+}$ was used to induce constructs in YEpCUP vectors.

*2.2. Plasmids*

The originals pGAL, pCUP [15] and YEp351 [16] were modified as follow: A *Nde*I-*Sma*I 1.04 kb fragment containing the *URA3* marker in pGAL was replaced with a 1.7 kb *Bam*HI fragment from YEp6 carrying the *HIS3* gene to give pGALHIS and with a 2.1 kb *Nar*I-*Hpa*I fragment from YEp351 carrying the *LEU2* gene to give pGALLEU. YEpCUPLEU was constructed by subcloning a 0.8 kb *Hin*dIII-*Bam*HI fragment carrying the CUP promoter and the CYC terminator from pCUP into the *Hin*dIII-*Bam*HI digested YEp351 vector from which the single Asp-718 site located in the polycloning cassette was previously eliminated. The YEpCUPTRP vector was constructed by replacing the 2.1 kb *Nar*I-*Hpa*I fragment containing the *LEU2* marker in YEpCUPLEU by a 1.2 kb *Eco*RI-*Pst*I fragment carrying the *TRP1* gene. YEpCUPLEU$\beta_1$ was constructed by ligating a 1.0 kb *Nco*I-*Hin*dIII fragment which carries the cDNA encoding for the G$\beta_1$ subunit (G$\beta_{36}$) of the human liver $G_s/G_i$ proteins [17] into the YEpCUPLEU vector previously digested with *Nco*I and Asp-718 after filling in both *Hin*dIII and Asp-718. Construction of YEpCUPLEU*STE4*$^{\Delta C6}$ was previously described [14]. YEpCUPTRP*STE4*$^{\Delta C6}$ was constructed by ligating the *Nco*I-Asp-718 fragment obtained from YEpCUPLEU*STE4*$^{\Delta C6}$ into the YEpCUPTRP vector previously digested with the same enzymes. Construction of pGALHIS*STE4* was described previously [14]. pGALLEU*STE4* was constructed by ligating an *Eco*RI-*Bam*HI fragment from pGALHIS*STE4* with pGALLEU partially digested with *Eco*RI and with *Bam*HI. Construction of pGALHISαs was done by ligating a 1.3 kb *Nco*I-*Hin*dIII fragment from a M13 clone that carries the cDNA encoding the $G_{\alpha s}$ subunit of the human liver stimulatory G protein [18] with the pGALHIS vector digested with *Eco*RI after filling in all ends with Klenow.

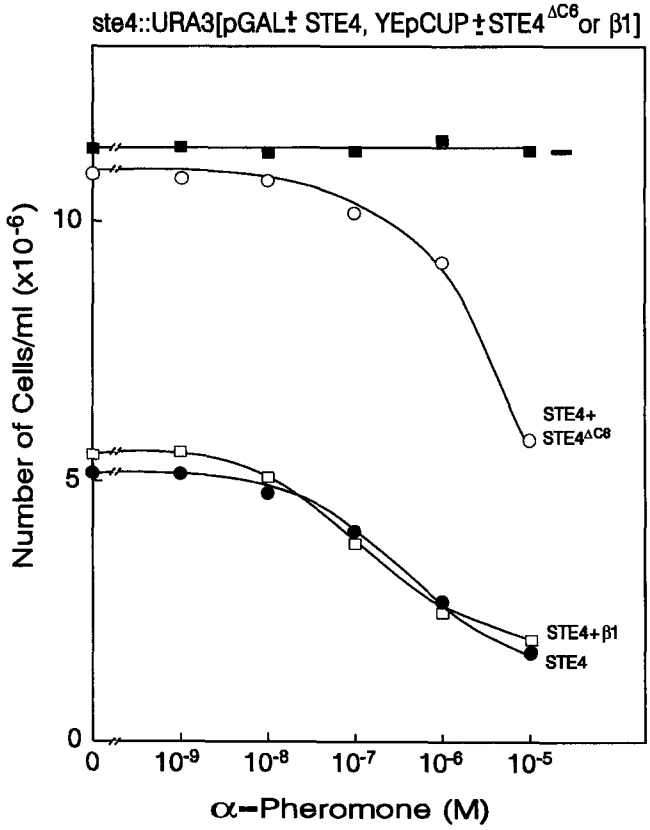


Fig. 1. Response to α-pheromone of *ste4* cells carrying different forms of *STE4* or mammalian $\beta_1$ in expression plasmids pGALHIS and YEpCUPLEU. *ste4* cells carrying the indicated plasmids were grown until midlog phase in SD plus the required amino acids. An aliquot of these cultures was transferred to SGal plus 100 μM $Cu^{2+}$ medium and incubated for 2 h at 30°C. $1 \times 10^6$ cells were transferred to 1 ml of fresh SGal plus 100 μM $Cu^{2+}$ containing different concentrations of α-pheromone and incubated for 6 h at 30°C with shaking. Cell number was determined using a cell counting chamber.

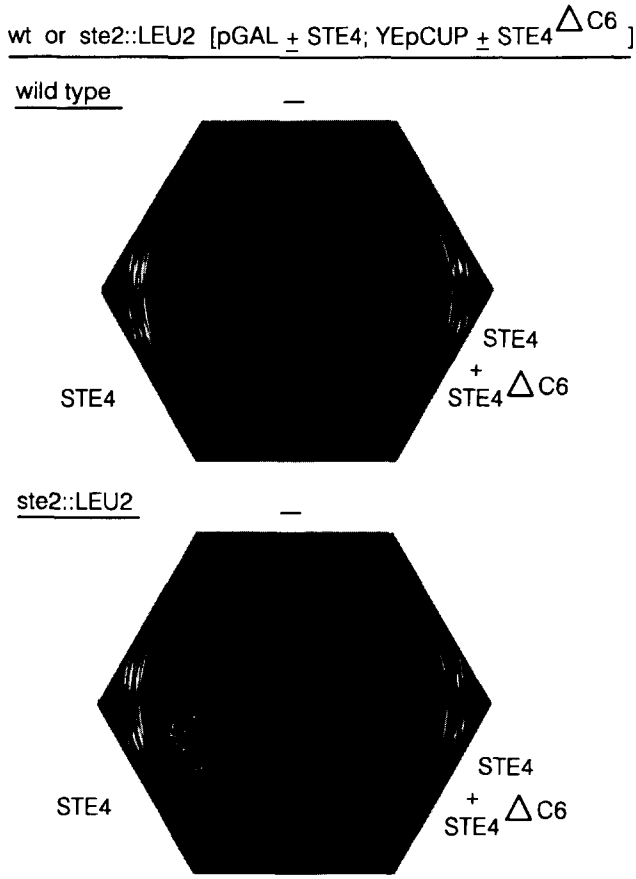


Fig. 2. Effect of STE4$^{\Delta C6}$ mutant on growth properties of wild type or *ste2* cells upon overexpression of STE4. *STE2* (upper panel) and *ste2* (lower panel) cells carrying the indicated plasmids were grown as indicated in Fig. 1. Cells were streaked onto a plate containing galactose and $Cu^{2+}$. Photographs were taken 48 h later.

*2.3. Growth arrest assays*

Cells were grown in SD medium containing the required amino acids. For liquid tests, $1 \times 10^6$ cells/ml were washed and transferred to SGAL medium containing 100 μM $Cu^{2+}$. Aliquotes were taken at different times and cells were scored in a cell-counting chamber. Growth arrest on plates was done by streaking cells on a SD plate and incubating at 30°C allowing single colonies to appear. One single colony was then streaked on a SGAL plate plus 100 μM $Cu^{2+}$ and incubated at 30°C. Pictures were taken after 48 h of incubation. Response to α-pheromone in liquid cultures was done as described previously [14].

*2.4. Miscellaneous*

Standard molecular biology procedures were as described by Sambrook et al. [19]. PCR clones were sequenced by the dideoxynucleotide chain-termination method using the Sequenace V.2.0 kit (United States Biochemical). Standard yeast genetics procedures were as described by Sherman et al. [20].

## 3. Results

Previous observations indicated that a STE4 subunit lacking six amino acids from its C-terminus is incapable of downstream signaling [14]. This was observed by expression of this truncated form in *ste4* and *ste2* mutants measuring mating competence and FUS1 induction. Co-expression of $\Delta$C6 mutant with the wild type subunit inhibit partialy the activity of STE4 and this effect of $\Delta$C6 was inhibited by excess STE18, indicating normal interaction between $\Delta$C6 and STE18. From these and other observations we conclude that the C-terminus of STE4 is essential for downstream signaling [14].

Addition of α-pheromone to *ste4* cells has no effect on their growth rate. Expression of high levels of STE4 (transcribed from pGAL plasmid) leads after a time of about 4 h to growth arrest. This effect is accelerated by α-pheromone in a dose-dependent manner with a half-maximal effect under our incubation conditions of $10^{-7}$ M in liquid culture (Fig. 1). While co-expression of an inactive protein (e.g. mammalian $G_{\beta 1}$; [14]) from a second plasmid did not per se interfere with the effects of STE4 and α-pheromone, expression under identical conditions of the C-terminally truncated STE4$^{\Delta C6}$ resulted in a blunting of the effects of STE4 and a right shift in the dose–response curve for the α-pheromone effect (Fig. 1).

We then explored if this dominant negative behavior of the

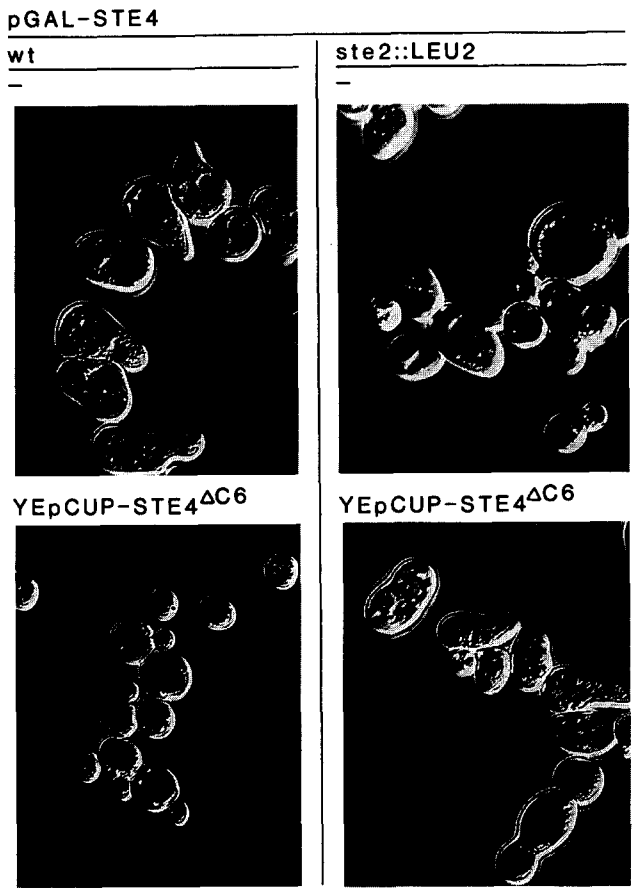


Fig. 3. Inhibition of induction of 'shmoo' morphology by STE4$^{\Delta C6}$ in wild type and *ste2* cells upon overexpression of STE4. Wild type (left panel) and *ste2* (right panel) cells carrying the indicated plasmids were grown as indicated in Fig. 1. Photomicrographs were taken using a Zeiss phase contrast microscope after 12 h of incubation at 30°C.

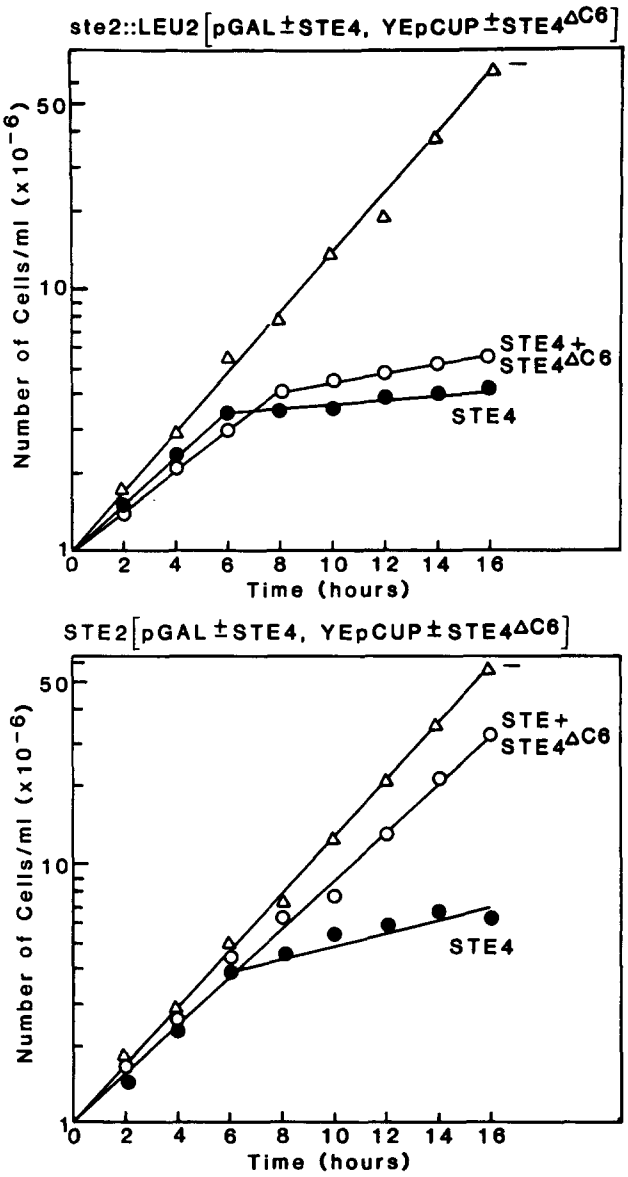


Fig. 4. Effect of overexpression of STE4 ± STE4$^{\Delta C6}$ on growth properties of *STE2* and *ste2* cells in liquid cultures. *ste2* (upper panel) and *STE2* (lower panel) cells carrying the indicated plasmids were grown as described in Fig. 1. Cell number was determined at different times.

STE4$^{\Delta C6}$ was dependent on the presence of the STE2 α-pheromone receptor. Experiments looking at growth on a SGal + $Cu^{2+}$ plate (Fig. 2), induction of shmoo morphology in liquid cultures (Fig. 3) and growth kinetics (Fig. 4) in receptorless *ste2 STE4* cells failed to show an effect of the $\Delta$C6 mutant of STE4. In contrast, when expressed in wild type cells (i.e. *STE2 STE4*) STE4$^{\Delta C6}$ inhibited the effect of overexpression of STE4 as seen in *ste4 STE2* cells.

Thus, even though our initial thoughts had been that the inhibition of STE4 action observed in *ste4* cells might have been due to a competitive interaction between STE4 and STE4$^{\Delta C6}$ at the level of the effector, the results obtained with *ste2* cells were incompatible with this hypothesis. That the inhibitory action of STE4$^{\Delta C6}$ required the STE2 receptor is striking and implicates an active role of the receptor in the blunting phenomenon.

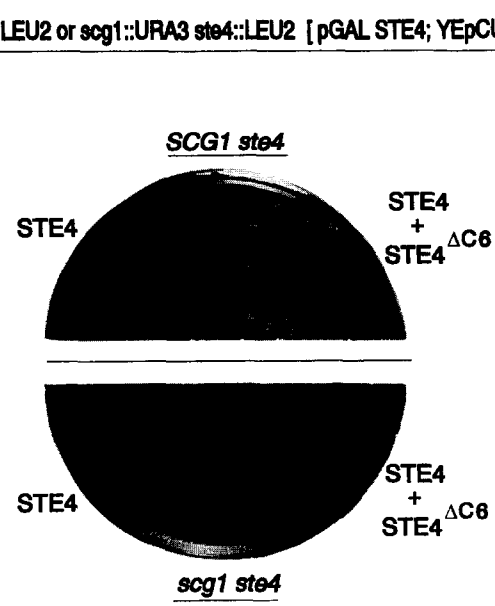


Fig. 5. Ability of STE4$^{\Delta C6}$ to inhibit the effect of overexpressing STE4 in *SCG1* and *scg1* backgrounds. A crossing was made between a *scg1* mutant carrying pGALHISαs, that would allow mating in glucose medium at low efficiency, and *ste4* mutant carrying pGALHIS*STE4*. Diploids were isolated by selection for lost of plasmids, sporulated and dissected. A segregant with the characters *ade1*, *his3*, *leu2*, *trp1*, *ura3*, *scg1::URA3*, *ste4::LEU2* was then selected. The *ste4* mutant and *scg1 ste4* double mutant carrying the indicated plasmids were grown as indicated in Fig. 1. Cells were streaked onto the surface SD medium and then replica-plated to SGal plus $Cu^{2+}$ and incubated at 30°C. Photographs were taken 48 h later.

Besides α-pheromone, the other elements known to interact with STE2 in *S. cerevisiae* are the subunits of the yeast G protein SCG1, STE4 and STE18. We thus explored whether the STE4 inhibiting effect of its ΔC6 mutant was dependent on SCG1. As shown in Fig. 5, in an *STE2* cell, the effect of ΔC6 depends on SCG1. A double mutant *scg1 ste4* was transfected with expression plasmid pGALHIS*STE4* alone or pGALHIS*STE4* plus YEpCUPTRP*STE4*$^{\Delta C6}$, and plated on selection media. Transfected cells were grown as patches onto selective media and replica-plated to media that would lead to expression of STE4 alone or STE4 plus its ΔC6 mutant. Growth of the *scg1 ste4* double mutant was compared with that of the *SCG1 ste4* cell, transfected with the same plasmids. As indicated in Fig. 5, the ΔC6 mutant failed to suppress the growth inhibiting action of STE4 in *scg1* cells in spite of the presence of the STE2 receptor.

## 4. Discussion

In our previous studies we showed that STE4$^{\Delta C6}$ competes with STE4 for STE18 and hence is competent to interact with the STE18 product [14]. The present studies show that inhibition of the growth arrest effect of STE4 by the ΔC6 mutant is not due to competition at the effector site, but rather involves an intrinsic activity of STE2 that is dependent on SCG1. These results are consistent with the existence of a basal pheromone-independent activity of STE2, which provides low levels of both an activated α (SCG1) and correspondingly low levels of βγ (STE4/STE18). For the βγ complex, these levels are not sufficient to trigger a response and require stimulation of STE2 through occupancy by pheromone to reach a critical concentration, or in the absence of pheromone require overexpression of STE4 by artificial means. In *STE2* cells not exposed to pheromone and expressing STE4 to form βγ, the basal levels of active SCG1 would then be sufficient to cooperate in some manner to potentiate or even allow for the growth arresting effect of βγ. If parallelisms can be drawn, it is worth noting that in mammalian G protein signalling systems, one of the hallmarks of βγ signalling is that it requires 100–1000 times higher concentrations of the dimer to trigger a response than are needed of an α subunit [21]. In this model of cooperative SCG1 and STE4 signalling, the activity of SCG1, like that of any G protein α subunit activated by GTP, is transient and lost upon hydrolysis of the GTP to GDP. It thus requires periodic renovation through interaction first with βγ to form α(GDP)βγ and then with STE2 to catalyze nucleotide exchange and activation. The most likely mechanism by which STE4$^{\Delta C6}$ can interfere with activation of trace amounts of active SCG1, is by formation of a poisoned heterotrimer composed of SCG1/STE4$^{\Delta C6}$/STE18 that binds to STE2 but cannot be acted upon to release active SCG1.

This model, in which the effect of STE4 to induce growth arrest and mating is postulated to depend on a cooperative effect of SCG1, predicts that STE4 should not be able to induce growth arrest in *scg1* cells, and hence that *scg1* should not be lethal. In fact the opposite is true, i.e. *scg1* cells are constitutively growth arrested even though they lack the SCG1 product postulated as a requirement for STE4 mediated growth arrest. This indicates that the real situation is more complex, with some other gene product(s) taking over the function of STE2/SCG1 when they are absent.

It may be that the function(s) that are interfered with by STE4$^{\Delta C6}$ relate to the fact that while *ste1 scg1(null)* cells enter spontaneously into growth arrest, their mating efficiency is very poor, only around 1% of the pheromone induced mating competence obtainable in wild type cells [8,11,22]. One possibility

is that activated SCG1 activates step 'X' and facilitates loss of CLN1. This may involve FUS3 and/or KSS1 [6]. In analogy with mammalian type I, II and IV adenylyl cyclases and mammalian type $\beta$2 phospholipase C, activated SCG1 may interact with the same effector as the STE4/STE18 $\beta\gamma$ complex, possibly STE20. Failure of STE2 SCG1 cells to enter into growth arrest in the presence of $STE4^{4C6}$ may serve as a useful screen to identify the putative positive effector of SCG1.

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