

Yeast α -mating factor receptor-linked G-protein signal transduction suppresses *Ras*-dependent activity

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Homologues of mammalian *Ras* conserved in *Saccharomyces cerevisiae* mediate glucose-stimulated cyclic AMP formation and we used this response to test for regulation of yeast *Ras* activity by the α -mating factor signal transduction pathway. α -Mating factor suppresses glucose-stimulated cyclic AMP formation by up to $57 \pm 12.6\%$ ($n=5$) and similar inhibition was observed in four different yeast strains (MATa cells). Moreover, this response is potent ($IC_{50}=0.14 \pm 0.19 \mu M$ ($n=4$)), rapid (maximal within 1–2 min), and displays an absolute requirement for both the α -mating factor receptor (STE2) and associated G-protein β -subunit (STE4). Inhibition appears independent of both phosphodiesterase activation and α -mating factor-stimulated cytoplasmic alkalinization. Also, basal cyclic AMP levels are unaffected by pheromone. This is the first demonstration that a cell-surface receptor linked to a heterotrimeric G-protein can suppress *Ras*-dependent activity and could provide important insight into mechanisms controlling p21^{ras} in man. Inhibition of *Ras*-dependent cyclic AMP formation could also be a key event facilitating responses characteristic of yeast mating.

Saccharomyces cerevisiae; α -Mating factor; *Ras*; G-protein; Cyclic AMP

1. INTRODUCTION

The *ras* gene family in mammalian cells includes Ha-*ras*, Ki-*ras* and N-*ras* which encode proteins of 21 kDa (p21^{ras}) displaying approximately 85% sequence similarity and which bind GTP and catalyse its hydrolysis to GDP. In most normal cells they are expressed at low levels on the inner surface of the plasma membrane where they are intimately involved in signalling pathways controlling proliferation and differentiation. Indeed, p21^{ras} proteins could be regulatory G-proteins mediating the effects of external growth factors. Moreover, activated *ras* oncogenes can transform mammalian cells in culture and have been implicated in the formation of a high proportion of human tumours [1–3]. The p21^{ras} proteins can switch between an inactive GDP-bound and active GTP-bound form with guanine nucleotide release proteins (GNRPs) promoting this interconversion by facilitating exchange of GDP for GTP [1]. Conversely, p21^{ras} inactivation follows GTP hydrolysis and GTPase activating proteins (GAPs) have been identified as components important for accelerating this process [1–4]. Despite the clear im-

portance of these regulatory proteins in neoplastic transformation, signal transduction pathways controlling p21^{ras} activation in response to extracellular signals have not yet been defined.

Homologues of mammalian p21^{ras} are conserved throughout all eukaryotic cells including the budding yeast *Saccharomyces cerevisiae* where extensive genetic evidence indicates that RAS1 and RAS2 gene products mediate rapid activation of adenylyl cyclase in response to stimulation by glucose [1–3,5,6]. In this system *Ras* activation through exchange of GDP for GTP is promoted by the protein product of the gene *CDC25*, while inactivation following GTP hydrolysis appears to be dependent upon components encoded by *IRA1* and/or *IRA2* [5–11]. These signalling elements appear analogous to the p21^{ras} system in mammalian cells and indeed human *ras* genes can replace disrupted yeast *ras* by ameliorating growth defects and by activating adenylyl cyclase [6,12]. Mutated yeast *Ras* can also transform mouse fibroblasts [13,14]. Moreover, expression of mammalian GAP in yeast suppresses phenotypes of *ira* mutants by reducing levels of GTP-bound *Ras* [11], while a C-terminal fragment of *SCD25* (a yeast protein structurally related to *CDC25*) catalyses guanine nucleotide exchange on human p21^{ras} [15]. Finally, as in mammalian cells, activation of yeast *Ras* is of central importance for promoting mitogenesis [5,6]. This high-degree of functional conservation between yeast and mammalian *Ras* and associated regulatory components suggests that study of *Saccharomyces cerevisiae* could give insight into molecular interactions important for controlling p21^{ras} in man.

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Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; G-protein, GTP-binding regulatory proteins; IC_{50} , concentration causing 50% inhibition

Recent data suggest that mammalian $p21^{ras}$ activity can be controlled through GAP following activation of the antigen receptor on T-cells [16] while $p21^{ras}$ in association with GAP also appears to block G-protein dependent control of atrial K^+ channel activity by muscarinic receptors [17]. These observations suggest a functional interaction between signalling pathways involving heterotrimeric G-proteins and the $p21^{ras}$ system. Since yeast possesses a well characterized α -mating factor receptor (encoded by *STE2* gene) linked to a G-protein consisting of α -, β - and γ -subunits (derived from genes *GPA1*, *STE4* and *STE18*), we tested whether this signalling pathway mediates 'crosstalk' modulation of *Ras*-dependent activity. The availability in yeast of adenylyl cyclase as a functional assay for *Ras*-activation together with a wide variety of defined mutants make this an ideal system in which to study an interaction between such G-protein systems and ultimately to describe unequivocally the key regulatory components involved.

2. MATERIALS AND METHODS

The following strains of *Saccharomyces cerevisiae* strains were employed: Sc 150 2B (MATa *leu2-3 leu2-112 ura3 trp1-289 his3-1*), NNY11 (MATa *trp1-289 ura3-52 leu2-3 leu2-112 lys2-801 his3-1*), NNY17 (NNY11 MATa $\Delta ste2::leu2$) [18], KMY2-11A (MATa *trp1 ura3 leu2 his3*), SNY5-1 (KMY2-11A MATa $\Delta ste4::leu2$) and BJ 1991 (MATa *leu2-3 leu2-112 pep4 ura3 trp1*). BJ1991 MATa cells carry identical markers to MATa cells of this strain.

All yeast strains were grown in YPD (2% glucose) at 30°C until mid- to late-log phase when cell cycle arrest at G_0/G_1 was induced by transfer to YP medium containing 0.02% glucose for 24 h. Cells were then washed twice in ice-cold water, resuspended in 25 mM MES (pH 6.0) at 30 mg/ml (approximately 5×10^8 cells/ml) and, at the times indicated after addition of 100 mM glucose (time zero), 250 μ l withdrawn and reactions terminated with 250 μ l of 16% TCA before freezing by immersion in dry-ice/methanol. Samples were then freeze-thawed 3 times and subsequently neutralized by 3 extractions with 4 vols. of water-saturated diethyl ether, freeze-dried, dissolved in 50 μ l of 50 mM Tris containing 4 mM EDTA (pH 7.5) and assayed for cyclic AMP using a kit from Amersham. To test for pheromone-dependent inhibition of glucose-stimulated adenylyl cyclase, yeast cells were exposed to α -mating factor added at time zero together with glucose. Intra-assay variability was <10% and all determinations were performed in duplicate (time courses) or triplicate (dose/response experiments). Percentage inhibition of maximal glucose-stimulated adenylyl cyclase was calculated at the time point giving peak cyclic AMP formation 1–2 min after exposure to sugar and α -mating factor.

3. RESULTS

Addition of 100 mM glucose to nutrient-deprived Sc 150 2B MATa cells resulted in a rapid increase in intracellular cyclic AMP which was maximal by 2 min. After this time cyclic AMP levels fell progressively although at 10 min remained 1.5- to 2.0-fold higher than basal (Fig. 1). Glucose-stimulated cyclic AMP formation confirms previously published observations of a response involving activation of *Ras*-dependent signal transduction [1,5,6,19,20]. Simultaneous addition of

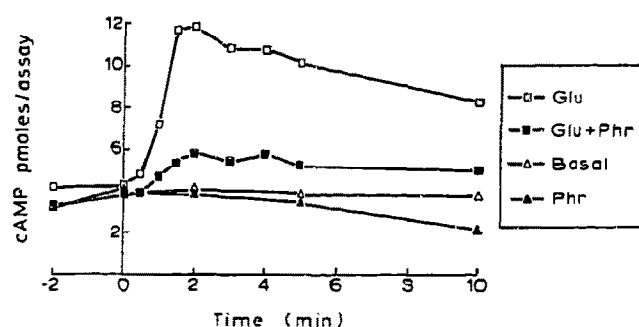


Fig. 1. Intracellular cyclic AMP levels measured in yeast strain Sc 150 2B (MATa) at times after adding 100 mM glucose (Glu). α -Mating factor (50 μ M) was added at time zero where indicated (Phr). Basal cyclic AMP levels (Basal) and the effect of 50 μ M α -mating factor acting alone are also shown. Yeast cells were growth-arrested by transfer to low-glucose (0.02%), stimulated with glucose and α -mating factor and aliquots containing 7.5 mg wet weight of yeast (approximately 10^8 cells) assayed for cyclic AMP as described in section 2. Points are the mean of duplicate determinations for one of the 5 identical experiments performed with this strain.

50 μ M α -mating factor at time zero resulted in a $57 \pm 12.6\%$ ($n = 5$) inhibition of maximal glucose-stimulated cyclic AMP formation. Basal cyclic AMP levels were unaltered by pheromone over the same time-course (Fig. 1). α -Mating factor-dependent suppression of glucose-stimulated cyclic AMP formation is likely to reflect inhibition of adenylyl cyclase activation rather than increased cyclic AMP degradation since IBMX at a concentration blocking endogenous yeast phosphodiesterase activity (1.0 mM) [21] does not diminish pheromone action (data not shown).

α -Mating factor has been reported to induce rapid transient intracellular alkalization within MATa yeast cells [22] and, given that cytosolic acidification stimulates *Ras*-dependent activation of adenylyl cyclase [7,23], this change in pH could suppress cyclic AMP formation. Sc 150 2B cell responsiveness was therefore tested either at pH 7.0 or in the presence of 1 mM 2,4-dinitrophenol (after 1 h preincubation) as these treatments abolish α -factor stimulated alkalization over the time course of peak glucose-stimulated cyclic AMP formation (>6 min) [22]. Hence, although under both these conditions glucose stimulated a characteristic sharp increase in cyclic AMP generation, 50 μ M α -mating factor was unaltered in its effectiveness as a suppressor of this response (data not shown). These observations suggest that α -mating factor-stimulated inhibition of *Ras*-dependent adenylyl cyclase activation is unlikely to be a secondary consequence of alkalization [22].

To confirm the specific involvement of the α -mating factor *STE2* receptor in this response, pheromone action was tested both by comparison in MATa and MAT α mating types and also using a *ste2*-null mutant (NNY17) [18]. Hence, while both MATa and MAT α

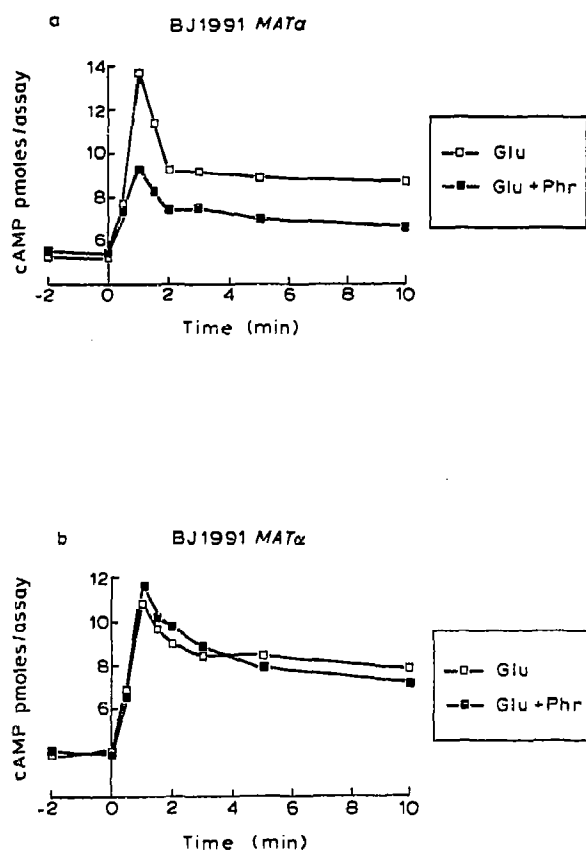


Fig. 2. Intracellular cyclic AMP levels measured in yeast strain BJ 1991 MATα (a) or MATα cells (b) at times after adding 100 mM glucose (Glu). α -Mating factor (50 μ M) was added at time zero where indicated (Phr). Yeast cells were treated as described in Fig. 1. Points are the mean of duplicate determinations for one of 6 identical experiments performed with this strain.

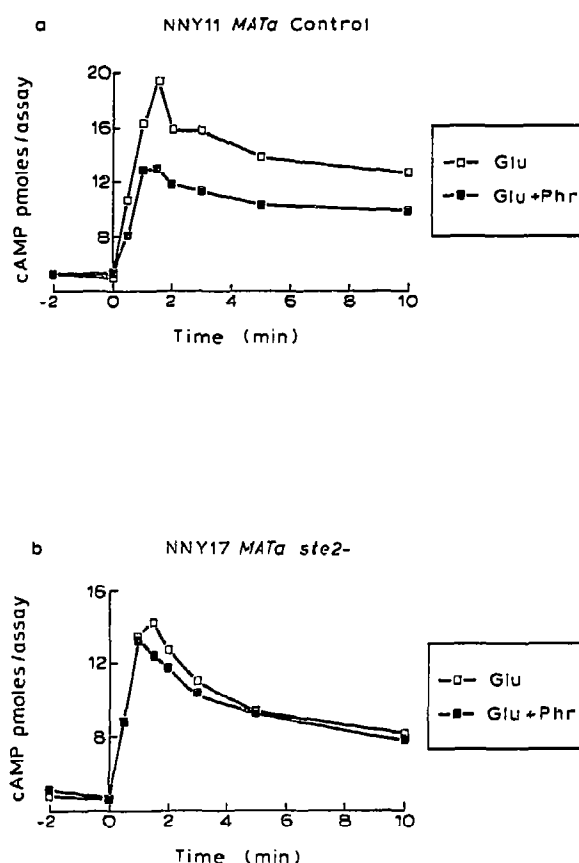


Fig. 3. Intracellular cyclic AMP levels measured in control NNY11 (MATα) (a) and *STE2*-receptor deficient NNY17 (MATα Δ ste2::leu2) (b) yeast cells at times after adding 100 mM glucose (Glu). α -Mating factor (50 μ M) was added at time zero where indicated (Phr). Yeast cells were treated as described in Fig. 1. Points are the mean of duplicate determinations for one of 7 identical experiments performed with these cells.

cells (strain BJ 1991) responded to 100 mM glucose with a sharp but transient rise in cyclic AMP concentration, 50 μ M α -mating factor was an effective inhibitor of this response in MATα cells only ($41 \pm 10.3\%$ ($n = 6$)) (Fig. 2a). Glucose-stimulated cyclic AMP formation was unaltered in MATα (Fig. 2b), an observation consistent with a dependency on the pheromone receptor since *STE2* expression is restricted to MATα cells [5,24]. To further confirm this absolute requirement for the α -mating factor receptor, experiments were performed using a mutant devoid of *STE2* (strain NNY17). Again, while both control and *ste2*-null mutant cells responded to 100 mM glucose with a rapid transient rise in cyclic AMP, 50 μ M α -mating factor was inhibitory only in control cells expressing the *STE2* receptor ($31 \pm 10.3\%$ ($n = 7$)). Mutant cells devoid of *STE2* were unresponsive to pheromone and gave a normal glucose response in the presence of 50 μ M α -mating factor (Fig. 3). Also consistent with involvement of *STE2* receptor, α -mating factor-dependent inhibition of glucose-stimulated adenylyl cyclase is potent and

saturable displaying an IC_{50} of $0.14 \pm 0.19 \mu$ M ($n = 4$) and maximal effects at 10 μ M (Fig. 4). This IC_{50} is close to the potency reported for pheromone-stimulated Ca^{2+} transport activity [25,26] and the K_d for [35 S] α -factor receptor binding to the *STE2* receptor in the presence of guanine nucleotide [27].

In yeast it is the G-protein β - γ dimer (*STE4*–*STE18*) which represents the active signalling component mediating the effects of α -mating factor [28–32]. To test whether this is also required for α -mating factor-dependent suppression of *Ras*-stimulated adenylyl cyclase activation we employed a mutant devoid of functional *STE4* (strain SNY5-1). While both control and *STE4* mutant responded with a characteristic rapid generation of cyclic AMP, inclusion of 50 μ M α -mating factor was an effective suppressor of this response in control cells only ($22 \pm 4.1\%$ ($n = 6$)) (Fig. 5). α -Mating factor was totally ineffective in mutant yeast devoid of *STE4* demonstrating that inhibition of *Ras*-mediated cyclic AMP formation is dependent upon the G-protein β -subunit.

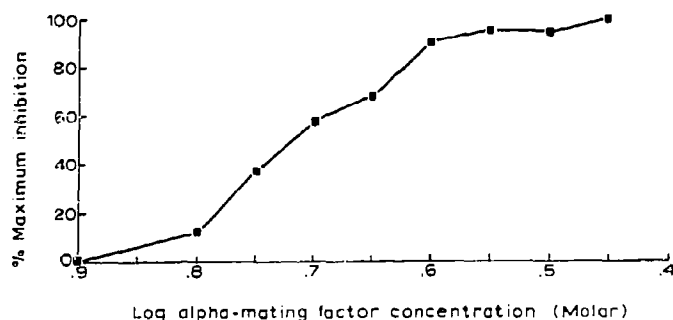


Fig. 4. Percent of maximum pheromone-dependent inhibition of glucose-stimulated cyclic AMP generation in the presence of increasing concentrations of α -mating factor in yeast strain BJ1991 (MATa). Inhibition was calculated at the time of peak cyclic AMP generation at 1–1.5 min after stimulation with glucose together with pheromone. Points are the means of 4 experiments each performed in triplicate and data yield an IC_{50} for pheromone action of $0.14 \pm 0.19 \mu\text{M}$.

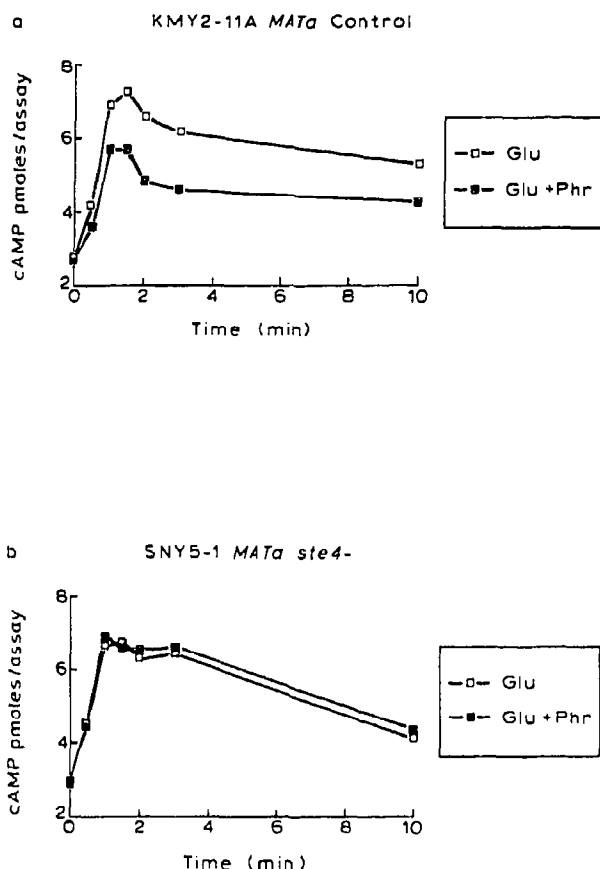


Fig. 5. Intracellular cyclic AMP levels measured in control KMY2-11A (MATa) (a) and mutant SNY5-1 deficient in G-protein β -subunit *STE4* (MATa $\Delta ste2::leu2$) (b) at times after adding 100 mM glucose (Glu). α -Mating factor ($50 \mu\text{M}$) was added at time zero where indicated (Phr). Yeast cells were treated as described in Fig. 1. Points are the mean of duplicate determinations for one of 6 identical experiments performed with these cells.

4. DISCUSSION

Mammalian $p21^{ras}$ is a class of low molecular weight G-protein intimately involved in cellular processes regulating mitogenesis and differentiation although pathways controlling its activation by extracellular signals are unknown [1–3]. Homologues of $p21^{ras}$ identified in *Saccharomyces cerevisiae* (encoded by *RAS1* and *RAS2*) display a high degree of structural and functional conservation with components in mammalian cells and also play an important role in mitogenesis. In addition, mechanisms regulating yeast *Ras* activation are similarly unclear [1–3,5,6]. Glucose-stimulated adenylyl cyclase represents a convenient measure of yeast *Ras*-activation [5–10] and we have employed this system to demonstrate an inhibitory ‘crosstalk’ following stimulation of the α -mating factor receptor-linked heterotrimeric G-protein. This response was observed in 4 different yeast strains, was rapid in onset (maximal within 1–2 min) and displayed an absolute requirement for both the *STE2* α -mating factor receptor and the *STE4* G-protein β -subunit. This is the first demonstration that receptor-linked activation of a heterotrimeric G-protein can suppress *Ras*-dependent signal transduction and could provide important future insight into mechanisms controlling activity of $p21^{ras}$ in mammalian cells. These data extend recent observations indicating novel mechanisms for activating $p21^{ras}$ involving GAP phosphorylation by either growth factor receptor-linked tyrosine kinase [1,2] or protein kinase C following stimulation of the T-cell antigen receptor [16] and demonstrate that *Ras* activity can also be under negative control by cell-surface receptors.

Despite the clear potential importance of inhibitory ‘crosstalk’ between heterotrimeric G-protein- and *Ras*-linked signal transduction pathways, the molecular interactions mediating this interplay are currently unknown. Indeed, although the insensitivity of basal cyclic AMP levels to α -mating factor indicates that adenylyl cyclase itself (encoded by *CDC35*) is not involved directly, other elements within this signalling system such as the CAP protein *SRV2*, *Ras*, *CDC25*, *IRA1* and/or *IRA2* all represent potential regulatory targets [5,6]. Important components within the mating response pathway are also unclear. Preliminary steps in the yeast mating response involves *STE2* receptor-stimulated dissociation of a heterotrimeric G-protein and convincing genetic evidence suggests that, in contrast to many mammalian systems, liberated β - γ dimer (*STE4*–*STE18*) is the active signalling component [28–32]. *STE4*–*STE18* activates additional regulatory products encoded by the genes *STE5*, *STE7*, *STE11* and *STE12* to elicit mating [24]. Although a role for such ‘downstream’ components or the G-protein α -subunit (*GPA1*) is not excluded, we have confirmed an absolute requirement for the β -subunit suggesting that *STE4*–*STE18* could also be a key intermediate for sup-

pression of glucose-stimulated cyclic AMP formation. Indeed, adenylyl cyclase inhibition may be similar to those identified in animal cells where one important mechanism involves receptor-dependent liberation of β - γ dimers from the inhibitory G-protein G_i which associate with free stimulatory G_s α -subunits to promote their inactivation [33]. In yeast *STE4-STE18* may be similarly able to combine with a G-protein α -subunit distinct from the *GPA1* gene product and which also facilitates *Ras* action. One possible candidate for this is an additional putative yeast G-protein α -subunit coded by the gene *GPA2* which has been identified previously as a potential adenylyl cyclase activator [34]. Studies are currently in progress to test this possibility and also define key molecular interactions involved.

Demonstration of inhibitory control of *Ras* activity raises important questions relating to the physiological events underlying mating in yeast. Hence, although extensive genetic studies have identified several key components in the mating response pathway (see above), the nature of the early biochemical signals generated following activation of the α -mating factor receptor are unclear [5,24]. Mating is characterized by cell-cycle arrest at 'START' in G_1 phase, cell wall synthesis, increased adhesiveness and morphological changes ('shmoo formation') and diminished cyclic AMP formation could represent a key event facilitating these responses. For instance, one consequence of cyclic AMP formation following glucose-stimulation is cell cycle progression [5,6] indicating that signal transduction by *Ras* and the α -mating factor receptor-linked G-protein are functionally antagonistic at the level of controlling mitotic division. Such a hypothesis is also consistent with reports that cell division arrest is accompanied by a decline in cyclic AMP levels [35] and that either exogenous cyclic AMP or elevation of endogenous levels in the presence of phosphodiesterase inhibitors can antagonize pheromone-stimulated mitotic arrest [21,36]. Despite this, α -mating factor effects appear specific for *RAS*-stimulated cyclic AMP formation as pheromone fails to diminish basal adenylyl cyclase activity in either membranes [37] or permeabilized cells [5,22], reports consistent with our observation that resting cyclic AMP levels remain unchanged for at least 5 min. Nevertheless, α -mating factor suppression of *RAS*-dependent cyclic AMP formation, together with other cellular events such as cytosolic alkalinization [22] and a slow increase in Ca^{2+} transport activity [25,26], could be an important signalling step mediating the full range of diverse and interactive responses characteristic of yeast mating.

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