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A constitutively active G-protein-coupled receptor causes mating self-compatibility in the mushroom *Coprinus*

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In the mushroom *Coprinus cinereus*, the multiallelic *B* mating type genes are predicted to encode a large family of seven-transmembrane domain receptors and CaaX-modified pheromones. We have shown that a single amino acid change Q229P in transmembrane domain VI of one receptor confers a self-compatible mating phenotype. Using a heterologous yeast assay, we have demonstrated that this *C.cinereus* pheromone receptor is a G-protein-coupled receptor and that the Q229P mutation is constitutively activating. A *C.cinereus* pheromone precursor was processed to an active species specifically in yeast *MAT*a cells and activated the co-expressed wild-type receptor. Yeast cells expressing the wild-type receptor were used to test the activity of synthetic peptides, enabling us to predict the structure of the mature *C.cinereus* pheromone and to show that the Q229P mutation does not compromise normal receptor function.

Keywords: constitutive receptor/*Coprinus*/GPCR/pheromones/yeast

Introduction

In filamentous basidiomycetes such as *Coprinus cinereus*, mating compatibility is determined by genes at two loci, designated *A* and *B* (see Casselton and Olesnicky, 1998). Each locus contains several multiallelic genes whose different combinations can determine many thousands of different mating types in natural populations. The *B* locus has recently been shown to contain genes encoding putative pheromones and receptors, indicating that pheromone signalling may play a crucial role in mate recognition (Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997; O'Shea *et al.*, 1998). Pheromone signalling is essential in the mating and sexual development of many fungi and, in general, pheromones are secreted to attract and prepare sexually compatible cells for fusion (reviewed by Bolker and Kahmann, 1993; Vaillancourt and Raper, 1996; Leberer *et al.*, 1997). The filamentous basidiomycetes are unusual, however, in that *B*-regulated development, and hence pheromone signalling, is initiated only after mating cells have fused (Swiezynski and Day, 1960; Raper, 1966).

Mating in *C.cinereus* converts an asexual mycelium (monokaryon) into a fertile mycelium, known as a dikaryon, on which the characteristic mushroom fruit bodies develop (reviewed by Casselton and Olesnicky, 1998). The dikaryon is a prolonged mycelial stage in which the nuclei from each mating partner remain together without fusing. To maintain both nuclei in each dikaryotic cell, a complex cell division occurs that requires the formation of a specialized structure known as a clamp connection. Both sets of mating type genes play a role in regulating the formation of this structure (Swiezynski and Day, 1960).

Incompatible matings in which monokaryons have different *A* alleles but the same *B* alleles give rise to mycelia with incomplete clamp connections. Haylock *et al.* (1980) used such mycelia to select for dominant mutations that overcame the incompatibility of the *B* alleles. Rare mutations were obtained that mapped to the *B* locus and were classed as self-compatible because they permitted strains to mate with monokaryons having the wild-type or mutant version of the same *B* locus. The role of the *B* genes in forming the clamp connections was constitutively induced in these mutants.

In this study we set out to characterize two self-compatible mutations in the *B6* locus. The *B6* locus is predicted to encode six pheromones and three receptors (O'Shea *et al.*, 1998) (Figure 1A). The sequences of the putative pheromone genes suggest that they encode long precursor molecules that end in a CaaX motif (C is cysteine, a is aliphatic and X is one of several amino acids), as found in the pheromones of other basidiomycete species (Bolker *et al.*, 1992; Moore and Edman, 1993; Spellig *et al.*, 1994) as well as **a**-factor of the budding yeast *Saccharomyces cerevisiae* (Michaelis and Herskowitz, 1988). The putative pheromone receptors in *C.cinereus* contain seven predicted transmembrane domains (O'Shea *et al.*, 1998) and show homology to the **a**-factor receptor (Ste3p) of *S.cerevisiae* (Hagen *et al.*, 1986). In yeast, the pheromone signalling pathway has been well characterized. Receptors couple to a heterotrimeric G protein to effect intracellular signalling through a MAP kinase cascade, leading to induction of genes required for mating (Kurjan, 1993; Herskowitz, 1995; Leberer *et al.*, 1997). Although we predict that a similar pheromone response pathway exists in *C.cinereus*, as yet none of the downstream components has been identified.

Using genetic experiments, we have shown that self-compatibility in both *B6* mutants is conferred by a receptor gene mutation. We have used yeast to provide a heterologous assay to demonstrate that this *C.cinereus* receptor acts as a G-protein-coupled receptor (GPCR) and that the self-compatible mutations cause its constitutive activation. In addition, we demonstrate that a *C.cinereus* pheromone precursor can be functionally processed in yeast *MAT***a** cells and can activate a co-expressed wild-type receptor. This has allowed us to predict the structure of a mature

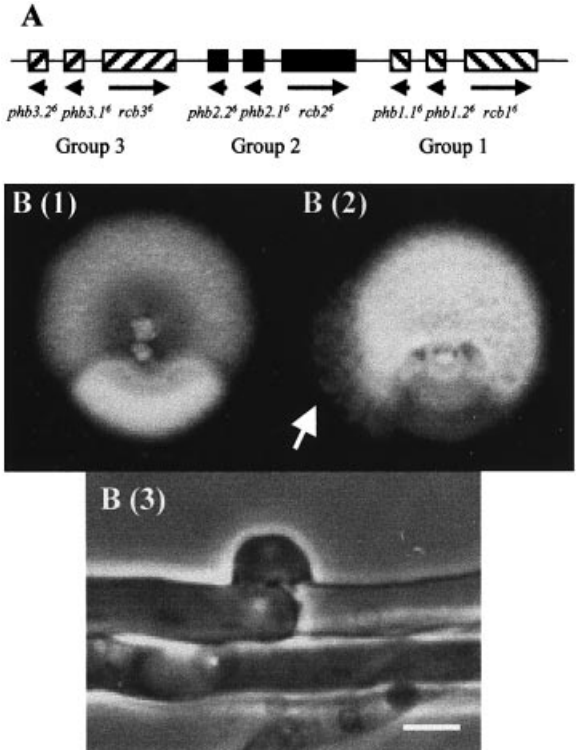

Fig. 1. Organization of the *C.cinereus B6* locus and the mating assay used to detect the self-compatible phenotype conferred by a single mutant gene within this locus. (**A**) Map of the *B6* locus based on O'Shea *et al.* (1998). Nine genes, three encoding receptors (larger boxes) and six encoding pheromones (smaller boxes), are arranged into three groups, designated 1, 2 and 3. Different shadings are used to indicate the three genes within each group that form a functional unit indivisible by recombination. (**B**) Mating assay used to distinguish wild-type and mutant genes. (1) An incompatible mating between the host strain (*A6B6*) transformed with wild-type *B*6 genes and tester strain (*A5B6*) that share the same *B* alleles. (2) A compatible mating between host transformed with the mutant *B*6 gene and the tester strain, giving rise to the dikaryon (arrowed). (3) Completed clamp connection on the dikaryotic cells. Scale bar, 5 μm.

C.cinereus pheromone and use a synthetic peptide to compare activation of the wild-type and mutant receptors.

Results

Mutations resulting in self-compatibility map to receptor genes

The wild-type *B6* locus is illustrated diagrammatically in Figure 1A. Each of the nine genes has previously been shown to activate *B*-regulated development when transformed into a *B3* host strain that contains different alleles of all the genes (O'Shea *et al.*, 1998). Not surprisingly, none of the genes activated development in a self *B6* host.

Each of the genes from a mutant *B6* strain (B6M1) was introduced individually into an *A6B6* host strain to test whether a single gene could confer the mutant phenotype. Because monokaryons in which the *B*-regulated pathway is active do not have a readily discernible morphology, the mutant phenotype could only be observed after performing a mating assay. Transformants were mated to a tester strain having a different *A* mating specificity, determined by different alleles at the *A* locus, but the same *B6* mating specificity (*A5B6*). The untransformed host, and hosts transformed with wild-type *B6* genes, were unable to form a dikaryon with the tester since the two strains have the same set of *B* genes [Figure 1B(1)]. In contrast, any transformant that expressed a dominant mutant gene was compatible with the tester [Figure 1B(2)], and formed a dikaryon with fused clamp connections [Figure 1B(3)]. Introduction of a single gene, *rcb2*^{6}, was sufficient to confer the self-compatible mutant phenotype (Table I). To test whether the mutation in the second mutant strain (B6M2) also resided in a receptor gene, the three receptor genes from this strain were isolated and tested in the same mating assay. The *rcb2*^{6} gene was again identified as the mutant gene responsible for the self-compatible phenotype. Both mutant genes were, like the wild type allele, capable of activating *B*-regulated development in a *B3* host (Table I).

The nucleotide sequences of both mutant *rcb2*^{6} alleles (designated *rcb2*^{6m}) contained a single base-pair substitution compared with the wild-type allele. The two mutations were found to be identical, and caused a glutamine to proline substitution, Q229P, towards the extracellular end of the sixth transmembrane domain of the predicted Rcb2^6 protein. Since these mutations were isolated independently, it was surprising that both led to the same base-pair change. However, Konopka *et al.* (1996) reported the repeated isolation of identical mutations after mutageniz-

Table I. Functional analysis of *B6* genes from wild-type and mutant strains

Origin	Gene	*B* Specificity in Host: Strain *B3*	Strain *B6*
B6M1	*phb3.2*^{6}		
B6M1	*phb3.1*^{6}		
B6M1	*phb2.2*^{6}		
B6M1	*phb2.1*^{6}		
B6M1	*phb1.2*^{6}		
B6M1	*phb1.1*^{6}		
B6M1	*rcb3*^{6}		
B6M1	*rcb2*^{6}		
B6M1	*rcb1*^{6}		
B6M2	*rcb3*^{6}		
B6M2	*rcb2*^{6}		
B6M2	*rcb1*^{6}		
H9	*rcb2*^{6}		

Genes from B6M1, B6M2 and wild-type H9 were introduced into two host strains, LCO12 (*A2B3, trp-3*) and LT2 (*A6B6, trp-1*), by co-transformation. Transformants were mated to tester strains PR94226 (*A6B3*) and FA2222 (*A5B6*), respectively. A clamp connection ( ) indicates that a portion of the transformants (>50%) had been co-transformed with a gene that changed *B* mating specificity and permitted dikaryon formation with the tester strain. No clamp connection ( ) indicates that none of the transformants had a changed *B* mating specificity.

ing the yeast α-factor receptor with hydroxylamine. It seems likely that the selection technique and the mutagen combine to favour isolation of specific mutations.

To confirm that the base-pair change detected by sequence analysis was sufficient to confer the mutant phenotype, a 210 bp *Bam*HI–*Eco*RI fragment containing the *rcb2*^{6m} mutation was exchanged with the equivalent fragment from the wild-type gene and vice versa. Both constructs generated were functional in a *B3* host, but, as predicted, only the gene containing the non-wild-type sequence activated *B*-regulated development in a self *B6* host. We conclude that the self-compatible phenotype is the result of a single missense mutation in the *rcb2*^{6} gene.

Rcb2^{6m} *is a constitutively active GPCR*

There are two obvious mechanisms by which the Q229P mutation in Rcb2^{6m} could cause a self-compatible phenotype. Either the mutation could allow the receptor to be activated by one of the normally incompatible pheromones encoded by the *B6* locus, or it could cause constitutive activation of the receptor so that it no longer required pheromone stimulation. To distinguish between these alternatives, a background was required in which *C.cinereus* receptors could be functionally expressed and to which pheromones could be added individually as required. In *C.cinereus*, no cellular background exists that is free of endogenous pheromones and receptors. We looked, therefore, for a suitable system in which to test receptor activity. The budding yeast *S.cerevisiae* was attractive as an experimental system as its pheromone response pathway is well characterized and has been engineered to provide functional assays for heterologous receptors whereby the pathway is linked to downstream reporter genes (King *et al.*, 1990; Price *et al.*, 1995). To activate this pathway, a receptor must couple to a heterotrimeric G protein composed of Gpa1p (G_α), Ste4p (G_β) and Ste18p (G_γ) subunits. Compatibility between receptors and G proteins involves specificity determinants at the C-terminus of the G_α subunit (reviewed by Conklin and Bourne, 1993). Not all heterologous receptors couple directly to yeast Gpa1p. However, chimeric G_α subunits in which the last five amino acids of Gpa1p are replaced by sequences from mammalian or fungal G_α proteins have been shown to change the specificity of Gpa1p without disturbing the signalling function of the heterotrimer (A.J.Brown and S.J.Dowell, unpublished).

In our initial experiments we used a *MAT***a** strain of *S.cerevisiae*, MMY8, in which the pheromone response pathway was linked to a reporter gene that conferred histidine prototrophy on activation. Wild-type and mutant *C.cinereus rcb2*^{6} and *rcb2*^{6m} cDNAs were expressed in MMY8 in combination with either Gpa1p or a chimeric G_α species. Transformants were grown in the presence of histidine, then transferred onto selective medium to test for a His$^+$ phenotype (Figure 2A). Growth on selective medium indicated that several of the chimeric G_α subunits were capable of coupling the mutant receptor to the pheromone response pathway. The best growth occurred in cells expressing chimeras of Gpa1p with either the promiscuous human G_α subunit, $G_{\alpha16}$, (Offermanns and Simon, 1995; Milligan *et al.*, 1996) or the Gpa3 subunit from another basidiomycete, *Ustilago maydis*, that has been implicated in the mating response pathway of this organism (Regenfelder *et al.*, 1997; Kruger *et al.*, 1998). Less efficient coupling was observed via Gpa1/$G_{\alpha s}$ and Gpa1p. No coupling was observed via a version of Gpa1p lacking the five terminal amino acids. Wild-type Rcb2^{6} also promoted low levels of histidine-independent growth in combination with the Gpa1/$G_{\alpha16}$ and Gpa1/Gpa3 chimeras, indicative of weak constitutive activity in the absence of pheromone. This is not unexpected, as many receptors exhibit constitutive activity when strongly expressed in a heterologous system (Milano *et al.*, 1994; Scheer and Cotecchia, 1997). However, the level of activity of the wild-type receptor was low when compared with the Rcb2^{6m} mutant receptor.

To quantify the extent of activation caused by the mutation, *rcb2*^{6} and *rcb2*^{6m} were expressed in strain MMY16, which expressed Gpa1/$G_{\alpha16}$ and in which the pheromone response pathway was coupled to a *lacZ* reporter gene. β-galactosidase assays confirmed that both receptors possessed intrinsic signalling capabilities in the absence of pheromone (Figure 2B). However, where the basal level of signalling exhibited by Rcb2^{6} was ~4-fold above background, signalling of the mutant receptor was >10-fold higher than the wild-type receptor.

To exclude the possibility that Rcb2^{6m} might be responding to the yeast pheromone produced by *MAT***a** cells (**a**-factor), receptor activity was examined in cells of the opposite mating type, *MAT*α, which do not produce **a**-factor. The HO endonuclease was used to induce a mating type switch in MMY8, generating the strain MMY8α. A control transformation using the yeast α-factor receptor (Figure 3A, *STE2*/vector) confirmed that the mating type switch had occurred and that the pheromone response pathway was active in MMY8α cells as the Ste2p receptor selectively responded to α-factor produced by these cells. MMY8α grew vigorously when expressing *rcb2*^{6m} (Figure 3A, *rcb2*^{6m}/vector), but relatively poorly with *rcb2*^{6} (Figure 3A, *rcb2*^{6}/vector). In a separate experiment, we tested whether the *C.cinereus* receptors responded to α-factor (Figure 2C). Neither Rcb2^{6} nor Rcb2^{6m} exhibited any response to synthetic α-factor, whereas Ste2p gave a robust response. We conclude that the activity of Rcb2^{6m} in yeast is due to constitutive activity rather than response to endogenous yeast pheromones.

A C.cinereus pheromone can be functionally expressed in S.cerevisiae

The constitutive activity of Rcb2^{6m} has allowed us to demonstrate that *C.cinereus* receptors can activate the yeast pheromone response pathway, and provides a potential assay for identifying natural ligands. The sequences of the putative *C.cinereus* pheromone precursors suggest that they belong to the same CaaX-modified family as yeast **a**-factor. We therefore examined whether a functional *C.cinereus* pheromone could be produced by yeast *MAT***a** cells, and whether this pheromone could be used to activate a *C.cinereus* receptor expressed in the same yeast cell.

Based on our genetic analysis of the *B6* locus (Casselton and Olesnicky, 1998; O'Shea *et al.*, 1998), we have argued that there are three functionally independent groups of genes (designated groups 1, 2 and 3 in Figure 1A), each comprising a receptor gene and two pheromone genes. Accordingly, we predict that activation of the *rcb2*^{6} receptor requires pheromones encoded by the group 2 phero-

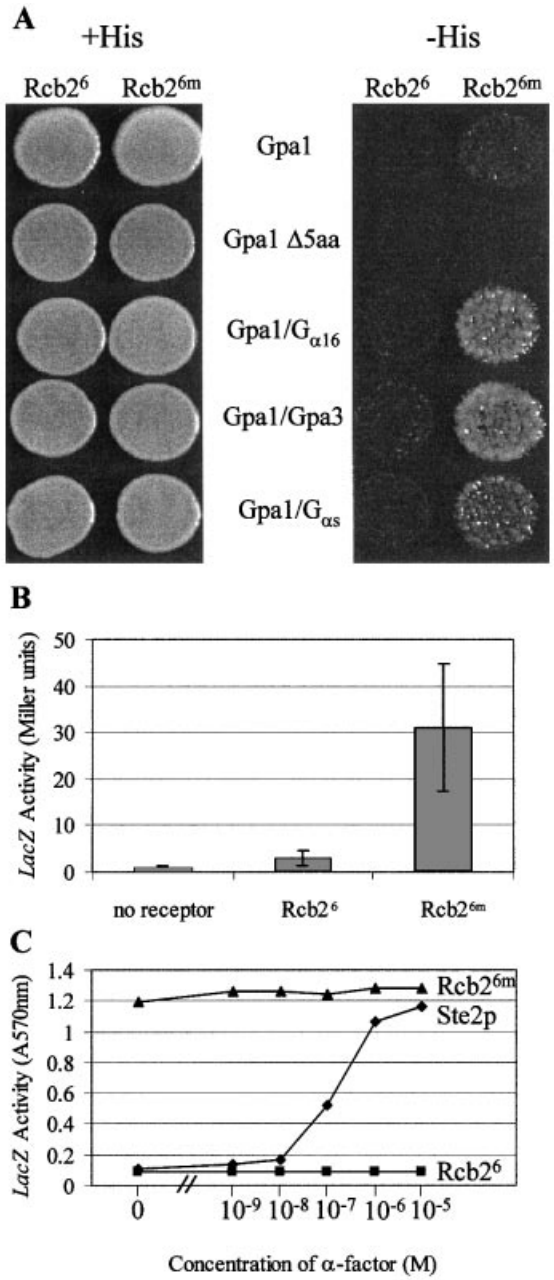

Fig. 2. Expression of the *C.cinereus* Rcb2^{6m} mutant receptor in yeast leads to constitutive activation of the pheromone response pathway. (**A**) Activation of the *FUS1–HIS3* reporter gene by Rcb2^{6} mediated by different G protein G_α subunits. Growth of MMY8 (*MAT***a**) cells expressing either the wild-type (Rcb2^{6}) or mutant (Rcb2^{6m}) receptor in combination with various G_α subunits was assessed on medium containing histidine (+His). Pathway activation was assessed on medium lacking histidine and supplemented with 10 mM 3-aminotriazole (3-AT) (–His). (**B**) Quantification of *FUS1–lacZ* reporter gene activation by Rcb2^{6} and Rcb2^{6m}. MMY16 cells expressing Gpa1/$G_{\alpha16}$ together with no receptor, Rcb2^{6} or Rcb2^{6m}, were incubated with the chromogenic β-galactosidase substrate *o*-nitrophenyl β-D-galactopyranoside (ONPG). Data are mean ± SD of six independent transformants. (**C**) Neither the wild-type Rcb2^{6} nor the mutant Rcb2^{6m} receptor is activated by yeast α-factor. MMY16 cells expressing Rcb2^{6}, Rcb2^{6m} or Ste2p were incubated with α-factor in the presence of chlorophenol red-β-D-galactopyranoside (CPRG). Conversion to the red product was determined after 24 h by absorbance at 570 nm. Data are the mean of two independent transformants.

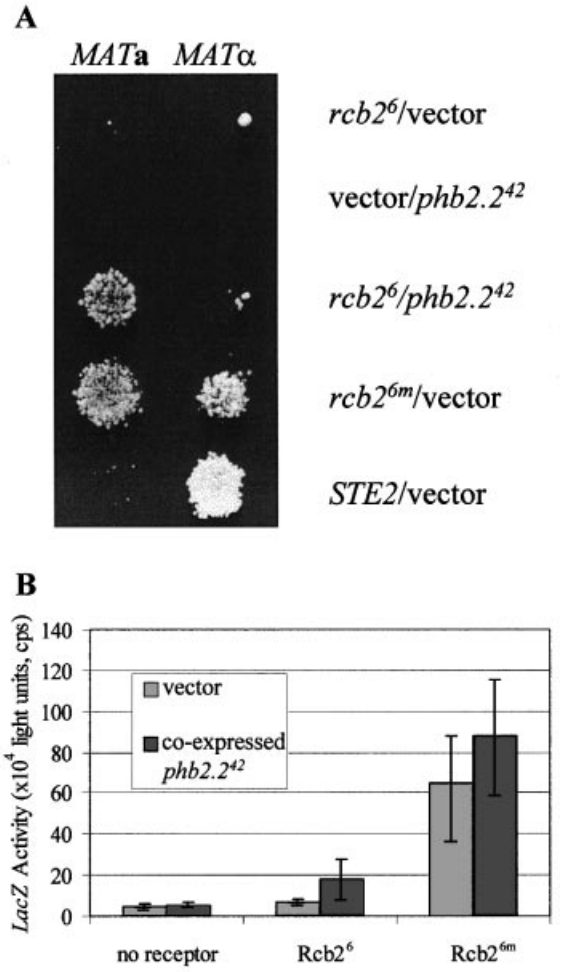

Fig. 3. Expression of the *phb2.2*^{42} pheromone precursor gene activates the Rcb2^{6} receptor. (**A**) MMY8 (*MAT***a**) and MMY8α (*MAT*α) cells co-transformed with various pheromone–receptor combinations or vector controls. *FUS1–HIS3* reporter gene activation was assessed on medium lacking histidine and supplemented with 10 mM 3-AT. (**B**) Stimulation of both wild-type and mutant Rcb2^{6} receptors by co-expressed Phb2.2^{42} pheromone. *FUS1–lacZ* activities were measured using a chemiluminescent assay in NSY3 cells expressing no receptor, Rcb2^{6} or Rcb2^{6m} in the presence of co-expressed Phb2.2^{42} or a control vector. Data are the mean ± SD of 12 independent transformants.

mone genes *phb2.1* and *phb2.2* (see Figure 1A) from a compatible locus. We have recently sequenced a second *B* locus, *B42*, which contains different alleles of these group 2 genes (Halsall, 1997 and unpublished) and which are known to activate *B*-regulated development in a *B6* strain. We chose *phb2.2*^{42} for this experiment. The gene, which encodes a 59-amino-acid precursor, was expressed in a *MAT***a** strain (NSY3) together with *rcb2*^{6} and *rcb2*^{6m}, and β-galactosidase activities were measured using a chemiluminescent assay (Figure 3B).

In the absence of compatible pheromones, the pattern of β-galactosidase induction paralleled that observed previously (Figure 2B), where signalling through the mutant receptor was significantly higher than it was through the wild-type receptor. Importantly, co-expression of Phb2.2^{42} had a small but measurable activating effect on both receptors, indicating that a functional *C.cinereus* pheromone can be produced in yeast and can activate both wild-type and mutant receptors in an autocrine manner.

This effect was most noticeable using the growth assay when co-expression of Phb2.2^{42} with Rcb2^{6} permitted strong growth on selective medium (Figure 3A).

An extensive series of *in vivo* modifications is involved in the production of a mature CaaX-modified pheromone (Caldwell *et al.*, 1995; Tam *et al.*, 1998). Post-translational modification involves farnesylation and carboxymethylation of the cysteine residue, and cleavage of the three C-terminal amino acids. The N-terminus of the precursor peptide is also cleaved to yield the mature pheromone (Caldwell *et al.*, 1995; Tam *et al.*, 1998). It is therefore noteworthy that *S.cerevisiae* can functionally process and secrete a *C.cinereus* pheromone. To determine whether the *C.cinereus* pheromone was being processed by the same pathway as yeast **a**-factor, Rcb2^{6} activity in the presence or absence of co-expressed Phb2.2^{42} was assessed in MMY8 and MMY8α using the growth assay. MMY8 expressing Rcb2^{6} and Phb2.2^{42} grew on histidine-deficient medium, but the corresponding *MAT*α transformants failed to grow, suggesting that these cells were not producing the pheromone required for receptor activation (Figure 3A, *rcb2*^{6}/*phb2.2*^{42}). This was significant because *MAT*α cells produce α-factor which is processed and secreted by a different pathway from **a**-factor (Kurjan, 1993). Cells of either mating type transformed with either receptor or pheromone genes alone failed to grow (Figure 3A, *rcb2*^{6}/vector and vector/*phb2.2*^{42}). Together, the results indicate that Phb2.2^{42} can be functionally expressed in yeast, specifically in *MAT***a** cells, supporting the hypothesis that the *C.cinereus* pheromone precursor is processed by the yeast **a**-factor maturation pathway and presumably secreted by the specialized Ste6p transporter.

Synthetic pheromones activate wild-type and mutant receptors in yeast

Although yeast could apparently process *C.cinereus* pheromones, the precise nature of the active species remained undefined. No pheromones have been purified from filamentous basidiomycetes. Indeed, it is not known whether pheromones in these species are secreted. Although N-terminal cleavage points cannot be deduced by comparison with those of other known fungal pheromone sequences, we have predicted them by comparing pheromone precursor sequences encoded by *B6* and *B42* genes (Halsall, 1997; Casselton and Olesnicky, 1998). Many of the precursors contain ER or DR residues 9–15 amino acids N-terminal to the cysteine of the CaaX motif, which may represent a potential cleavage site that would generate mature peptides of a size similar to those isolated from other fungi (see Bolker and Kahmann, 1993; Vaillancourt and Raper, 1996). Five peptides of different lengths were synthesized based on the predicted sequence of mature Phb2.2^{42}. Each peptide was C-terminally carboxymethylated. In addition, a farnesylated version of each peptide was synthesized (Figure 4A).

MMY16 cells expressing wild-type Rcb2^{6} receptor were incubated in the presence of the 10 peptides. The farnesylated peptide of 12 amino acids (#4F), with the intact ER motif, strongly activated the receptor (Figure 4B). The farnesylated peptide of 13 amino acids (#5F) and the unfarnesylated versions of both peptides (#4 and #5) could also activate the receptor, but to much lesser extents. That unfarnesylated *C.cinereus* pheromones should be active confirms previous results where a mutant pheromone containing a C-terminal arginine residue, rather than the cysteine with its concomitant modifications, was shown to be weakly active *in vivo* (O'Shea *et al.*, 1998).

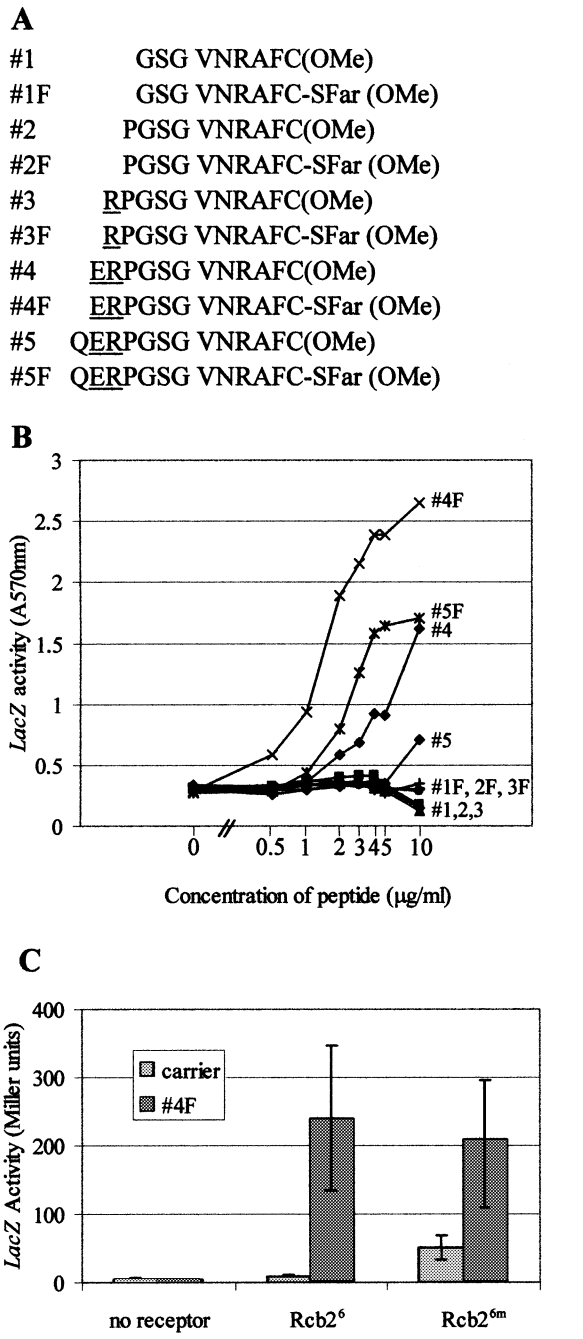

Fig. 4. Synthetic peptides activate wild-type and mutant Rcb2^{6}. (**A**) Structures of the 10 synthetic peptides tested for activation of Rcb2^{6} receptors. The C-terminal residue of each peptide was carboxymethylated, and the peptides designated #1F to #5F were also farnesylated, via the sulfhydryl group of the cysteine residue. The conserved Glu–Arg (ER) motif is underlined. (**B**) Relative agonist potencies of synthetic peptides measured in MMY16 cells expressing Rcb2^{6}. The *FUS1–lacZ* response to peptides was measured using the CPRG assay. Data values are representative of results from three independent experiments. (**C**) Rcb2^{6} and Rcb2^{6m} activation by peptide #4F. *FUS1–lacZ* activities were measured using an ONPG assay in MMY16 cells expressing either no receptor, Rcb2^{6} or Rcb2^{6m} in the presence of 1.5 μg/ml peptide #4F or carrier. Data are the mean ± SD of three independent transformants.

None of the shorter farnesylated peptides could activate the receptor. In contrast, truncations of up to four amino acids from yeast **a**-factor possess residual biological activity against the Ste3p **a**-factor receptor (Caldwell *et al.*, 1994). We conclude that the farnesylated dodecapeptide containing the ER motif most likely corresponds to mature Phb2.2[42].

The response of wild-type and mutant receptors to synthetic peptide #4F was measured in MMY16 (Figure 4C). The synthetic pheromone activated Rcb2[6] 25-fold over the basal level in the absence of pheromone. Interestingly, the mutant receptor was stimulated to approximately the same level, suggesting that this represents maximum activity of Rcb2[6]. Thus, the mutation that renders Rcb2[6m] constitutively active does not appear to prevent pheromone binding nor to affect the receptor's signalling capacity in the presence of pheromone.

Discussion

In the basidiomycete *C.cinereus*, evolution has determined that a haploid monokaryon can mate with almost any other. The ability to distinguish self from non-self is in part determined by the specificity contained in an individual's complement of pheromones and receptors encoded by genes of the *B* mating type locus. Mutations that confer self-compatibility compromise a system that has evolved to maximize genetic diversity in the population and, not surprisingly, such mutations are very rare (Haylock *et al.*, 1980). In this study we have identified a mutant receptor as the cause of the self-compatible phenotype in two *C.cinereus* strains. We have shown by sequence analysis that the receptor has a single amino acid substitution and have used functional assays to demonstrate that this structural alteration results in constitutive activity in the absence of pheromone stimulation.

Due to the large family of receptors and pheromones present in the natural cellular environment, we could not assay the function of the mutant Rcb2[6m] receptor in *C.cinereus*. We chose to do this in a yeast system that has been optimized for the study of heterologous receptors. By expressing Rcb2[6m] in yeast we confirmed for the first time that *C.cinereus* receptors can function as G-protein-coupled receptors and provided the experimental evidence that the mutant receptor has constitutive activity.

Rcb2[6m] has a single amino acid substitution at the extracellular end of the predicted transmembrane domain (TMD) VI, a characteristic of mutations in some constitutively active mammalian GPCRs. Mutations in which a threonine at the extracellular end of TMD VI is replaced with a proline have been reported to result in constitutive activation of glucose-dependent insulinotropic peptide receptor (Tseng and Lin, 1997) and m5 muscarinic receptor (Spalding *et al.*, 1995). In addition, Spalding *et al.* (1997) have described a series of replacements of a serine residue at the end of TMD VI in the m5 muscarinic receptor that cause constitutive activation. A change within TMD VI is likely to have a structural effect consistent with models for receptor activation that implicate a movement of TMD VI relative to other helices (Bourne, 1997; Scheer and Cotecchia, 1997; Dube and Konopka, 1998).

The *C.cinereus* pheromone precursors have a CaaX sequence which suggested a structure similar to yeast **a**-factor, but the precise length of mature pheromones could not be deduced from the gene sequence alone. Our data suggest that the conserved ER motif is a likely processing recognition site, with cleavage occurring directly N-terminal to the glutamate. In the autocrine system, active pheromone was only produced in *MAT***a** cells, suggesting that pheromone processing requires the **a**-factor synthetic machinery. Significantly, application of synthetic pheromone confirmed that Rcb2[6] was functional in both *MAT***a** and *MAT*α cells (data not shown). Farnesylation is essential for the processing and export of **a**-factor (Caldwell *et al.*, 1995) and since this modification greatly enhanced the activity of the synthetic *C.cinereus* pheromones, it is likely that the endogenous pheromones are synthesized by a similar pathway to **a**-factor. The low level of receptor activation by autocrine expressed *C.cinereus* pheromone relative to synthetic peptide may be due to inefficient or inappropriate processing, or competition with **a**-factor for processing. Since synthetic pheromone was able to activate Rcb2[6] and Rcb2[6m] to equivalent maximal levels, the Q229P mutation does not prevent ligand binding or the process of receptor activation, but is likely to shift the receptor conformational equilibrium towards an activated state.

The yeast assay was essential to determine the structure of an active pheromone since no assay system exists to measure the activity of exogenously applied ligands in *C.cinereus*. In this regard, the synthetic farnesylated peptide that is clearly a potent agonist of the *C.cinereus* receptors in the yeast assay did not promote fusion of clamp cells nor induce transcription of pheromone genes when applied to growing *C.cinereus* hyphae (data not shown). Tests with *C.cinereus* culture filtrates provided no evidence of a secreted natural pheromone that could activate *B*-regulated development. Since pheromone secretion plays no role in *C.cinereus* mate attraction, pheromone binding may not occur on the outer surface of hyphal cells, and the receptors may have a specific subcellular localization not accessible to pheromones in the culture medium. However, we cannot rule out the possibility that the peptide that is active in the yeast assay may differ in structure from the natural pheromone produced in *C.cinereus* cells.

The yeast assay we have established to study the function of the mutant *C.cinereus* receptor has considerable potential for studying other aspects of pheromone-receptor recognition in this fungus. Genetic studies have led to the prediction that each *B* locus of *C.cinereus* encodes three functionally independent groups of pheromones and receptors (Casselton and Olesnicky, 1998; O'Shea *et al.*, 1998), a system that allows for the generation of a predicted 79 different *B* mating specificities in natural populations. Remarkably, a given pheromone can activate many receptors and a given receptor can be activated by many pheromones, with specificities being determined by minor differences in primary sequence. Yeast provides us with the tools to investigate structure–function relationships within this large family of genes, which will ultimately yield important information about the activities of GPCRs and their ligands in other systems.

Materials and methods

Coprinus strains and growth conditions

Coprinus cinereus strains used in this study were: H9 (*A6B6* wild type), LT2 (*A6B6 trp-1.1;1.6*), FA2222 (*A5B6 trp-1.1;1.6*), B6M1 (*A5B6m1*

ade-5), B6M2 (*A2B6m2 ade-5 ade-8 met-5*), LCO12 (*A2B3 trp-3*), PR94226 (*A6B3 ade-5 cho-1*), PG78 (*A6B42 pab-1 trp-1.1;1.6*) and LN118 (*A42B42 ade-2 trp-1.1;1.6*). Media and methods for culturing *C.cinereus* were described by Lewis (1961), with modifications described by Mutasa *et al.* (1990). Hosts for transformation were LT2 and LCO12. *B6* genes were introduced by co-transformation with plasmids pCc1001 or pDB1 containing the *C.cinereus trp-1* and *trp-3* genes, respectively. Transformation was performed as described by Casselton and De La Fuente Herce (1989). Routinely, 50 transformants were tested for the expression of an introduced *B6* gene. Frequency of co-transformation was variable, but a positive mating reaction was always detected in 20% of transformants.

B gene plasmid generation

The nine *B* genes from the B6M1 strain (previously referred to as BM5) were isolated as described by O'Shea *et al.* (1998). The three receptor genes from the B6M2 strain were amplified by PCR using the following primers: *rcb1*^{6}, CCCCGAACGGCCTTGTACTGTAGC and CTCGCTCTGCTCCCGGACC; *rcb2*^{6}, AAGCTTGGGGCGGACGATGCG and AAGCTTAGTAAGAGGACATGAGTCCC; and *rcb3*^{6}, GGATCCTTGGACGGGGAAGAGGACGG and CCCGCGTTTCTTGGAGCCG. The wild-type *rcb2*^{6} gene was amplified from the H9 genomic DNA using *rcb2*^{6} primers as for strain B6M2. The PCR products were cloned into pGEM-T (Promega). Both wild-type and mutant *rcb2*^{6} were also amplified using high-fidelity *Pfu* polymerase (Stratagene) to confirm the nucleotide sequence. DNA sequence was obtained using the PRISM^{TM} Ready Reaction DyeDeoxy^{TM} Terminator Cycle Sequencing Kit (Perkin Elmer) for analysis on an ABI 373 automated sequencer (Perkin Elmer).

RNA for RT–PCR was isolated (O'Shea *et al.*, 1998) from LN118 transformed with *rcb2*^{6m} from B6M1. The cDNA was generated using Superscript^{TM} II RNaseH-reverse transcriptase (Gibco-BRL) using primer GCGCGCAAGCTTAACGGCCGACCATGGTTCGGGGTTGG. The cDNA was amplified using *Pfu* polymerase and primer CCCGGGCTTAAGATGGCTCCCTCTCACGAC. The PCR product was cloned into pUC18 (Promega). A wild-type *rcb2*^{6} cDNA was generated via subcloning from the genomic sequence.

A vector to express heterologous receptors in yeast was created by removing the α-factor prepro leader sequence of pPIC9 (Clare *et al.*, 1991) and introducing it adjacent to the promoter in p416GPD (Mumberg *et al.*, 1995) to create p416GPDleader. The cDNA encoding *rcb2*^{6} was cloned into p416GPDleader as an in-frame fusion with the α-factor prepro leader to direct expression to the secretory pathway (Romanos *et al.*, 1995).

The *phb2.2*^{42} gene was amplified from a cloned LN118 sequence using *Pfu* polymerase (Stratagene) and the primers GGATCCTACAAAATGGATAATTTCACCGTCG and AAGCTTTTAGGCGATCACACAAAATGCGC. The PCR product was cloned into the yeast expression vector p425GPD (Mumberg *et al.*, 1995). The synthetic pheromones were generated using a C-terminal carboxymethylated cysteine residue, which was farnesylated after peptide synthesis was complete (Babraham Institute, Cambridge).

Yeast strains, manipulations and G_α and Ste2p expression constructs

Yeast strain MMY8 was derived from W303–1A (*MAT***a** *his3 ade2 leu2 trp1 ura3 can1*) by a series of one-step gene replacements (Rothstein, 1998) using *gpa1*Δ*::ADE2*, *far1*Δ*::URA3* and *sst2*Δ*::URA3* DNA constructs. The *ura3* mutation was recovered by sequential transformations with a *ura3*Δ fragment consisting of *URA3* with an internal 243 bp (*Eco*RV to *Stu*I) deletion, and 5-fluoro-orotic acid selection. The *FUS1–HIS3* reporter gene was integrated at the *FUS1* locus. Mating type conversion of MMY8 was carried out as described by Guthrie and Fink (1991) using YEp13-HO (Jensen *et al.*, 1983) to generate MMY8α. Strain MMY9 was derived from MMY8 by integrating the *FUS1–lacZ* reporter gene at the *leu2* locus as described (Nomoto *et al.*, 1990). Strain MMY11 was derived from MMY9 as described (Wach, 1996) using a *ste2*Δ*::G418*^{R} fragment generated by PCR with primers GCTCTGGCTATAATTATAATTGGTTACTTAAAAATGCACCGTTAAGAACC ATATCCAAGAATCCCCCCTGCGTACGCTGCAGGTCGAC and CTAGTAGTAACCT TATACCGAAGGTCACGAAATTACTTTTTCAAAGCCGTAAATTTTGGAATCGATGAATTCGAGCTCG. The *lys2*Δ*::FUS1–lacZ* disruption cassette was produced by PCR from YIpFUS102 (Nomoto *et al.*, 1990) using primers TATTTAAATTATTGTACATGGACATATCATACGTAATGCTCAACCGAAATGGATACGGATAAGTTAATC and AAACTGCTAATTATAGAGAGATATCACAGAGTTACTCACTATTATTATTATTTTTGACACCAGACC. NSY1 was generated by transforming *lys2*Δ*::FUS1-lacZ* into MMY8, selecting on α-aminoadipic acid and screening by a blue colony assay as described by Dowell *et al.* (1998) for integrants in which the pheromone response pathway was activated.

Plasmid pJW1 was derived from pRS314 (Stratagene) by insertion of the *GPA1* promoter (1 kb), a polylinker (*Stu*I, *Bam*HI, *Nru*I, *Cla*I, *Nco*I, *Sma*I, *Xho*I, *Bgl*II, *Not*I) and the *ADH1* terminator. Plasmid pRS314-GPA1 to express wild-type Gpa1p was created by inserting the *GPA1* open reading frame into the *Nru*I site of pJW1. To generate Gpa1/G_α chimeras, the *Afl*II site in the *GPA1* promoter of pRS314-GPA1 was removed by blunt-ending with Klenow and religation. Codon 467 of *GPA1* was mutated (Quikchange, Stratagene) from AAA to AAG, creating an *Afl*II site, and *Afl*II/*Xho*I oligonucleotide linkers were inserted. Resulting plasmids encoded Gpa1p truncated of the C-terminal five residues (Gpa1$^{1–467}$), or fusions between residues 1–467 of Gpa1p and the five C-terminal acids of mammalian G_α subunits $G_{\alpha s}$ (QYELL^{COOH}) or $G_{\alpha 16}$ (EINLL^{COOH}), or the *Ustilago maydis* G_α subunit (DSGIL^{COOH}) (Regenfelder *et al.*, 1997). To integrate Gpa1/$G_{\alpha 16}$ into the yeast genome, a cassette comprising the *GPA1* promoter, Gpa1/$G_{\alpha 16}$, and the terminator was cloned to pRS304 (Sikorski and Hieter, 1989). Strains MMY16 and NSY3 were derived from MMY11 and NSY1, respectively, by integration of plasmid pRS304-Gpa1/$G_{\alpha 16}$ at the *trp1* locus. Routine yeast manipulations were performed according to Guthrie and Fink (1991). To permit expression of *STE2* in *MAT*α cells, the coding sequence was amplified using the primers GCCGTAAAAAGCTTTCAGCGGCCGAATAAATTATTATTATCTTCAGTCCAG and CCATATCCGAATTCACCATGTCTGATGCGGCTCCTTCATTGAGC and cloned into pPGK (Kang *et al.*, 1990) adjacent to the *PGK1* promoter to generate pPGK–STE2.

Assays of β-galactosidase activity in yeast cells

Assays of β-galactosidase activity using the chromogenic substrate chlorophenolred-β-D-galactopyranoside (CPRG; Boehringer) were performed by suspending cells at 0.02 OD_{600}/ml in SC medium lacking amino acids to select for plasmid maintenance in 96-well flat-bottomed microplates (200 μl per well). Medium was supplemented with 10 mM 3-aminotriazole, 0.1 mg/ml CPRG and 0.1 M sodium phosphate pH 7. After incubation at 30°C for 24 h, absorbance (570 nm) was determined in a Victor plate reader (Wallac). In competition assays, cells were preincubated for 1 h at 37°C with synthetic pheromone peptides in medium lacking CPRG, before performing the assay. Assays using the chemiluminescent Galacton-Star substrate were performed by suspending cells in 100 μl SC-WH medium in 96-well microplates. After incubation (30°C; 6 h), 20 μl aliquots were added to a 20 μl assay mix [125 mM sodium phosphate pH 7.5, 15 mM $MgSO_4$, 200 μM Galacton-Star substrate (Tropix), 10% (v/v) Sapphire II (Tropix), 1 U/μl oxalyticase (Enzogenetics)]. After 1 h at 30°C, chemiluminescence was determined in a Top-count scintillation counter (Packard). Assays using the substrate ONPG were performed as described by Dowell *et al.* (1998).

Sequence data accession No.

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AF136522 and Y11081 for *phb62.2*^{42} and *rcb2*^{6}, respectively.

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