Self-Compatible *B* Mutants in Coprinus With Altered Pheromone-Receptor Specificities

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ABSTRACT

A successful mating in the mushroom *Coprinus cinereus* brings together a compatible complement of pheromones and G-protein-coupled receptors encoded by multiallelic genes at the *B* mating-type locus. Rare *B* gene mutations lead to constitutive activation of *B*-regulated development without the need for mating. Here we characterize a mutation that arose in the *B6* locus and show that it generates a mutant receptor with a single amino acid substitution (R96H) at the intracellular end of transmembrane domain III. Using a heterologous yeast assay and synthetic pheromones we show that the mutation does not make the receptor constitutively active but permits it to respond inappropriately to a normally incompatible pheromone encoded within the same *B6* locus. Parallel experiments carried out in Coprinus showed that a F67W substitution in this same pheromone enabled it to activate the normally incompatible wild-type receptor. Together, our experiments show that a single amino acid replacement in either pheromone or receptor can deregulate the specificity of ligand-receptor recognition and confer a self-compatible *B* phenotype. In addition, we use the yeast assay to demonstrate that different receptors and pheromones found at a single *B* locus belong to discrete subfamilies within which receptor activation cannot normally occur.

MATING compatibility in the homobasidiomycete fungus *Coprinus cinereus* is determined by several multiallelic genes that reside at the *A* and *B* mating-type loci. Provided mates have different alleles of both sets of genes, somatic cell fusion is sufficient to initiate a morphogenetic program that converts an asexual monokaryotic mycelium into a fertile dikaryotic mycelium on which mushroom fruit bodies develop (see Raper 1966; Casselton and Olesnicky 1998).

The dikaryon represents an extended mycelial stage between cell fusion and nuclear fusion. Each cell of the dikaryon contains two nuclei, one from each mate, and a compatible complement of mating-type genes ensures that this equal nuclear distribution is maintained until a late stage in fruit body differentiation when the nuclei fuse. Following cell fusion, there is exchange and migration of nuclei through the established cells of each monokaryon. Once the tip cells contain genetically different nuclei, a complex cell division follows in which the two nuclei divide in synchrony and a structure known as a clamp connection is formed through which one of the daughter nuclei must pass into a newly formed subterminal cell. Compatible *A* genes are required for synchronized division of the nuclei and for the formation of the clamp cell. Compatible *B* genes promote the initial nuclear migration that establishes binucleate cells and, later, control fusion of the clamp cell to the subterminal cell to complete the clamp connection (Swiezynski and Day 1960).

The *B* mating-type genes, the subject of this report, encode lipopeptide mating pheromones and their G-protein-coupled receptors (GPCRs; Wendland *et al.* 1995; Vaillancourt *et al.* 1997; O'Shea *et al.* 1998). Pheromone signaling is known to play an important role in mating in other fungi, and typically, pheromones are secreted to attract compatible mates and prepare cells for fusion. The mushroom fungi are unusual in that cell fusion occurs independent of pheromone signaling, and signaling appears to be important only for regulating the postfusion events of nuclear migration and clamp cell fusion. The mushrooms are, moreover, unique among the fungi in having a large family of mating pheromones and receptors. In *C. cinereus*, two *B* loci have been sequenced, *B6* and *B42*, and both contain nine multiallelic genes (O'Shea *et al.* 1998; Halsall *et al.* 2000). Genetic studies indicate that these constitute three functionally redundant sets of genes, each set encoding a receptor and two pheromone precursors. These different sets of genes are paralogous and

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encode three discrete subfamilies of signaling molecules that are unable to activate each other. Analysis of strains with different *B* mating specificities reveals that these are generated from different allele combinations of the three sets of genes, with an estimated 79 unique combinations in nature (Halsall *et al.* 2000). In another homobasidiomycete, *Schizophyllum commune*, there appear to be only two sets of genes (Wendland *et al.* 1995), but each has nine alleles, which together generate a predicted 81 *B* mating specificities (Raper 1966).

The complexity in the *B* gene families makes *C. cinereus* and *S. commune* attractive models to study receptor-ligand interactions. A single receptor may be activated by many different ligands and a single ligand can activate several different receptors. Of particular interest, since GPCRs are ubiquitous in eukaryotic cells, are mutations that alter receptor-ligand specificity since they may help us to better understand regions of the molecules that determine this. *B* gene mutations are known in both mushroom species and were obtained long before the functions of the genes were elucidated by selecting for *B*-regulated development in the absence of a compatible mate (Parag 1962; Koltin 1968; Haylock *et al.* 1980). All the mutations obtained were dominant and mapped to the *B* locus.

Haylock *et al.* (1980) isolated three independent mutations in the *B6* locus of *C. cinereus*. We recently characterized two of these mutations, showing that both caused an identical amino acid substitution (Q229P) in one of the three *B6* receptors and resulted in constitutive activity of the receptor (Olesnicky *et al.* 1999). Functional analysis of this receptor was not possible in *C. cinereus* because its activity could not be assessed in the absence of the six pheromones that the *B6* locus also encodes. To overcome this, we developed a heterologous assay in which the *C. cinereus* receptors were expressed in *Saccharomyces cerevisiae*. The attraction of this organism is that its pheromones and their receptors are well characterized, few in number, and can be readily deleted by gene disruption. Moreover, modifications have been established that enable foreign receptors to be coupled to the yeast pheromone response pathway (Brown *et al.* 2000). Activation of *C. cinereus* receptors in yeast was coupled to expression of a reporter gene, allowing us to characterize the mutant receptor. Importantly, this system allowed us to predict the structure of a mature *C. cinereus* pheromone. The genes encode precursor molecules of some 50–75 amino acids that are, like *S. cerevisiae* **a**-factor, post-translationally modified to give much shorter peptides that are carboxymethylated and farnesylated (see Caldwell *et al.* 1995). Precursor sequences are highly variable both within and between families. Each, however, has a carboxy-terminal CaaX motif and a conserved Glu Arg/Asp Arg (ER/DR) motif upstream of which is the predicted recognition site for proteolytic cleavage to give mature peptides of 12–14 amino acids. By predicting the active pheromone structure we have been able to carry out reproducible assays of receptor activation using purified synthetic peptides.

In this study we use the yeast assay and appropriate synthetic peptides to show that the third mutation identified by Haylock *et al.* (1980) generates a different class of receptor mutant, one that responds to a normally incompatible pheromone encoded at the same *B* locus. We also demonstrate that a mutant pheromone can cause self-compatibility by making directed amino acid changes that confer the ability to activate a normally incompatible wild-type receptor.

MATERIALS AND METHODS

***C. cinereus* strains and growth methods:** *C. cinereus* strains used were LT2 (*A6B6 trp-1.1;1.6*); FA2222 (*A5B6 trp-1.1;1.6*); B6M3 (*A2B6m2 ade-8 met-5, ade5*); PG78 (*A6B42 pab-1 trp-1.1;1.6*); J65,5, *A43B42*. Media and methods for culturing *C. cinereus* were described previously (Olesnicky *et al.* 1999). Hosts for transformation were LT2 and PG78. *B6* genes were introduced into LT2 and PG78 by cotransformation with plasmid pCc1001 containing the *C. cinereus trp-1* gene. Transformation was performed as described by Casselton and de la Fuente Herce (1989). Routinely, 50 transformants were tested for expression of the introduced *B6* gene. Frequency of coexpression ranged from 20–80% of transformants tested. *B* gene function was assayed using the mating test described by O'Shea *et al.* (1998). Transformants were crossed to tester strains having a different *A* mating specificity but the same *B* mating specificity as the transformed host. Activation of *B*-regulated development in the host by a compatible *B* gene made the transformant compatible in mating with the tester and resulted in formation of the characteristic dikaryotic mycelium.

Construction of vectors for epitope tagging: A *Bgl*II/*Bam*HI linker encoding the hemagglutinin (HA) epitope flanked by an ATG start codon was generated by annealing the oligonucleotides GATCTTGAAAATGTACCCATACGACGTCCCAGA CTACGCTCG and GATCCGAGCGTAGTCTGGGACGTCGT ATGGGTACATTTTCAA. This linker was phosphorylated, treated with T4 DNA ligase, and digested with *Bgl*II and *Bam*HI. A 126-bp fragment comprising three copies of the HA epitope fused in frame was cloned into pBB (Dowell *et al.* 1998) between *Bgl*II and *Bam*HI sites. This fragment was subcloned into the *Bam*HI site of p426GPD (Mumberg *et al.* 1995) to generate p426HA1, in which the triple HA epitope is under the transcriptional control of the glyceraldehyde-3-phosphate gene (*TDH1*) promoter. Next, p426HA1 was subjected to mutagenesis (Quikchange; Stratagene, La Jolla, CA) using the primer pairs CCAGACTAC GCTCGGGATCCCCCGGGCTG CAGGAATTCG and CGAATTCCTGCAGCCCGGGGGATCC CGAGCGTAGTCTGG and CCAGACTACGCTCGGGGATCC CCCGGGCTGCAGGAATTCG and CGAATTCCTGCAGCCC GGGGGATCCCCGAGCGTAGTCTGG. These introduced one and two nucleotides, respectively, to alter the frame of the polylinker and to generate p426HA2 and p426HA3. Hence, protein-coding DNA may be inserted into the polylinker (*Bam*HI, *Sma*I, *Pst*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Cla*I, *Sal*I, *Xho*I) of an appropriate version of this vector to generate an in-frame, N-terminal extension comprising three copies of the HA epitope tag.

***B* gene plasmid generation:** The three receptor genes from the B6M3 strain were amplified by PCR using the following primers: *rcb1*^{6}, CCCCGAACGGCCTTGTACTGTAGC and CT CGCTCTGCTCCCGGACC; *rcb2*^{6}, AAGCTTGGGGCGGACGAT

GCG and AAGCTTAGTAAGAGGACATGAGTCCC; and *rcb3*^{6}, GGATCCTTGGACGGGGAAGAGGACGG and CCCGCGTTTC TTTCTTGGAGCCG. PCR products were cloned and sequenced as described previously (Olesnicky *et al.* 1999). For expression in yeast, cDNA sequences encoding the wild-type Rcb3^{6} and Rcb3^{6} R96H receptors were generated by RT-PCR using the primers CCCTTTGTATCAGCAGCAGACATCTT TATGCTTTAACATCTGT and CCGTAGAGACAGATGTTAA AGCATAAAGATGTCTGCTGATACAAAGGG. cDNAs were first cloned into pUC18 and subcloned into p416GPDlinker (Olesnicky *et al.* 1999), to generate p426GPD-Rcb3^{6} and p426GPD-Rcb3^{6}R96H, and also into p426HA1. RNA for RT-PCR was derived from a *B42* strain (PG78) that had been transformed with Rcb3^{6} or Rcb3^{6} R96H, since receptor gene expression is upregulated by the pheromone response. Synthetic pheromone Phb2.2^{42} was described by Olesnicky *et al.* (1999). Phb3.2^{6}, Phb3.2^{42}, and Phb2.2^{6} were synthesized similarly (Severn Biotechnology, Kidderminster, UK). The yeast expression construct p426GPDleader-Rcb2^{6} containing the Rcb2^{6} cDNA was described previously (Olesnicky *et al.* 1999). The R95H mutation was introduced into p426GPD-Rcb2^{6} (Quikchange) using oligonucleotides CCCTTTGTAT CAGCAGACATCTTTATGCTTTAACATCTGTCTCTACGG and CCGTAGAGACAGATGTTAAAGCATAAAGATGTCTGC TGATACAAAGGG. Mutations in *phb3.2*^{6} were generated by inverse PCR (Imai *et al.* 1991). Two primers containing the wild-type sequences, GTGAGTGCGCCCTGAAT and GGAGG CAATGGCCTTACCT, were modified or extended appropriately to generate the following codon changes: CAC (H) to CAA (Q); AAT (N) GGT (G): TTC (F) to TGG (W); TGG (W) to TCT (F); and TTCTGG (FW) to TGGTCT (WF).

Yeast strains and methods: Yeast strains MMY8, MMY11, and MMY16 and the chimeric G-protein α-subunit Gpa1/$G_{\alpha i1}$ have been described previously (Olesnicky *et al.* 1999; Brown *et al.* 2000). To integrate Gpa1/$G_{\alpha i1}$ into the yeast genome, a cassette comprising the *GPA1* promoter, Gpa1/$G_{\alpha i1}$, and the *ADH1* terminator was cloned into pRS304 (Sikorski and Hieter 1989). Strain MMY23 was derived from MMY11 by integration of pRS304-Gpa1/$G_{\alpha i1}$ at the *trp1* locus. Assay of β-galactosidase activity using the chromogenic substrate chlorophenolred-β-d-galactopyranoside (CPRG; Boehringer Mannheim, Indianapolis) was performed as described previously (Olesnicky *et al.* 1999). Data are mean ± standard deviation of four independent transformants. Western blotting was performed as described by Brown *et al.* (2000) using purified anti-HA monoclonal antibodies (16B12) obtained from Babco.

RESULTS

Mutation in Rcb3^{6} confers a self-compatible *B* mating phenotype: The organization of the *B6* and *B42* loci as determined by O'Shea *et al.* (1998) and Halsall *et al.* (2000) is summarized in Figure 1. Each locus contains nine genes, organized into three discrete groups designated 1, 2, and 3, each comprising a receptor gene and two pheromone precursor genes. The predicted compatible pheromone-receptor combinations are indicated. While the two pheromone genes associated with each receptor are generally similar in sequence, they are not identical and do not always display the same spectrum of activity toward a particular receptor as seen for Phb3.1 and Phb3.2. The mutation in the B6M3 strain isolated by Haylock *et al.* (1980) was localized to the *rcb3*^{6} gene, which encodes the group 3 receptor, by transformation experiments. Genes from the *B6* locus of B6M3 were isolated by PCR and introduced into a *B6* host strain of *C. cinereus* (LT2, *A6B6*). This host has the wild-type *B6* genes and introduction of the dominant mutant gene conferred the self-compatible phenotype. Self-compatibility was detected by the ability of the transformed host to mate with strains having the normally incompatible self *B6* mating specificity (O'Shea *et al.* 1998; Olesnicky *et al.* 1999). Sequencing of the mutant, *rcb3*^{6m}, identified a single G to A transition that would cause an arginine to histidine substitution (R96H) at the junction of the third transmembrane domain (TMD III) and the second intracellular loop of the predicted Rcb3^{6} protein (Figure 2A). A 360-bp *Pfl*M1-*Bsi*WI restriction fragment from *rcb3*^{6m} containing this mutation was used to replace the corresponding fragment in wild-type *rcb3*^{6}. This chimera induced a self-compatible mating interaction when introduced into a wild-type *B6* host. In contrast, replacement of this fragment in the mutant gene by the corresponding wild-type gene sequence abolished self-compatibility (Figure 2B). We conclude that the mutant phenotype was a direct consequence of this single base substitution in the receptor gene.

In our previous analysis of *B6* self-compatible mutants we identified the mutation in the group 2 receptor Rcb2^{6}. The mutant Rcb2^{6} Q229P was able to activate

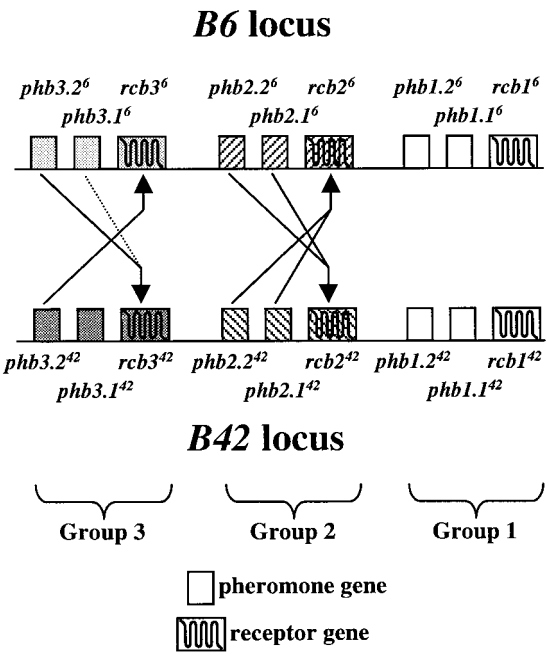

Figure 1.—Diagrammatic representation of the organization of the *B6* and *B42* mating-type loci of *C. cinereus* showing that each contains three groups of genes. As indicated by the shading, *B6* and *B42* are heteroallelic for the group 2 and group 3 genes but homoallelic for the group 1 genes. Compatible pheromone/receptor gene combinations, based on the genetic data of O'Shea *et al.* (1998) and Halsall *et al.* (2000), are indicated by arrows. The dotted line indicates that the interaction is weak.

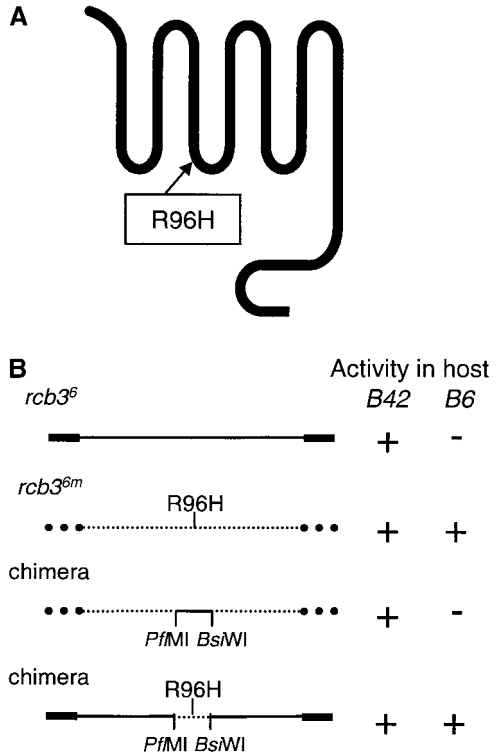

FIGURE 2.—A single amino acid substitution in a pheromone receptor causes mating self-compatibility. (A) Position of the predicted R96H replacement in the Rcb3^6 receptor at the junction of TMD III and the second intracellular loop. (B) Transformation of a wild-type *B6* strain with the mutated form of rcb3^6, *rcb3^{6m}* (R96H), confers self-compatibility in matings with other *B6* strains of *C. cinereus*. All four versions of the *rcb3^6* gene confer self-compatibility on the *B42* host strain.

the pheromone response pathway of a heterologous *S. cerevisiae* host independent of pheromone (OLESNICKY *et al.* 1999). We carried out similar experiments to determine if Rcb3^6 R96H was also constitutively active. Complementary DNAs encoding Rcb3^6 and Rcb3^6 R96H were introduced into the yeast strain MMY8, which contains the *FUS1-HIS3* reporter gene to allow growth on histidine-selective media dependent on activation of the pheromone response pathway. As in our previous experiments (OLESNICKY *et al.* 1999) we tested the endogenous yeast G-protein α-subunit, Gpa1, as well as a series of chimeric G_α subunits, to optimize the efficiency of G-protein coupling to the *C. cinereus* receptor.

Neither wild-type Rcb3^6 nor Rcb3^6 R96H receptor stimulated histidine-independent growth of MMY8 yeast cells (data not shown). To confirm that this apparent lack of constitutive activation of Rcb3^6 R96H was not due to lack of expression or coupling to the G_α subunits tested, we demonstrated that Rcb3^6 could be activated by a synthetic pheromone. Genetic studies in *C. cinereus* (HALSALL *et al.* 2000) enable us to predict that Rcb3^6 is activated by Phb3.2^{42}, a pheromone encoded in the *B42* locus (see Figure 1). We predicted that the mature Phb3.2^{42} pheromone would be a farnesylated carboxymethylated 14-mer peptide ERRTQGGGGLTWFC-SFar-(OMe). Yeast cells expressing either wild-type Rcb3^6 or Rcb3^6 R96H were incubated with synthetic Phb3.2^{42} in the presence of the β-galactosidase substrate CPRG. Conversion of this substrate to a red product is indicative of *FUS1-lacZ* reporter gene induction due to activation of the signal transduction pathway.

No induction of *FUS-lacZ* occurred in the absence of synthetic Phb3.2^{42}, either with Rcb3^6 or Rcb3^6 R96H, confirming that, when expressed in yeast, the R96H mutation does not cause detectable constitutive activation of the receptor. However, when the synthetic pheromone was applied to cells expressing wild-type Rcb3^6, a concentration-dependent response was observed, demonstrating that Rcb3^6 was expressed and could couple to the yeast pheromone response pathway (Figure 3). We performed this experiment using a panel of different yeast strains expressing various wild-type and chimeric G_α subunits to determine which yielded the most efficient coupling of Rcb3^6 to the pheromone response pathway (BROWN *et al.* 2000). Maximal coupling was achieved with the Gpa1/$G_{\alpha i1}$ transplant chimera, in which the five C-terminal amino acids of Gpa1p are replaced with the C-terminal residues of mammalian $G_{\alpha i1}$. The results presented in Figure 3 were obtained using coupling to this Gpa1/$G_{\alpha i1}$ chimera. Remarkably, cells expressing the mutant Rcb3^6 R96H receptor also responded to the synthetic pheromone but with a greater sensitivity than the wild-type receptor, detecting lower concentrations of Phb3.2^{42} and giving increased *FUS1-lacZ* activation at high ligand concentrations.

Rcb3^6 R96H is activated by Phb3.2^6, a *B6* (self) pheromone: We considered several explanations for the increased ligand sensitivity of yeast expressing Rcb3^6 R96H. Given the location of the mutation in the second intracellular loop of the receptor, it seemed unlikely that the residue at this position would contact the pheromone directly. However, it may influence G-protein coupling, it may have an indirect effect on ligand binding, or it may cause greater levels of receptor protein to accumulate by stabilizing the receptor or by disrupting a mechanism of desensitization. To investigate this, we generated epitope-tagged versions of Rcb3^6 and Rcb3^6 R96H in which the receptor N terminus was fused to a peptide sequence containing three copies of the HA epitope. The epitope tag marginally reduced the efficiency of coupling of both HA-Rcb3^6 and HA-Rcb3^6 R96H. However, HA-Rcb3^6 and HA-Rcb3^6 R96H both retained the ability to respond to synthetic Phb3.2^{42} pheromone, and the phenotype of enhanced coupling of Rcb3^6 R96H was also observed with the tagged receptors (Figure 3A). Levels of HA-Rcb3^6 and HA-Rcb3^6 R96H protein in yeast whole-cell extracts were quantified by immunoblotting with anti-HA monoclonal antibodies and were indistinguishable (Figure 3, B and C).

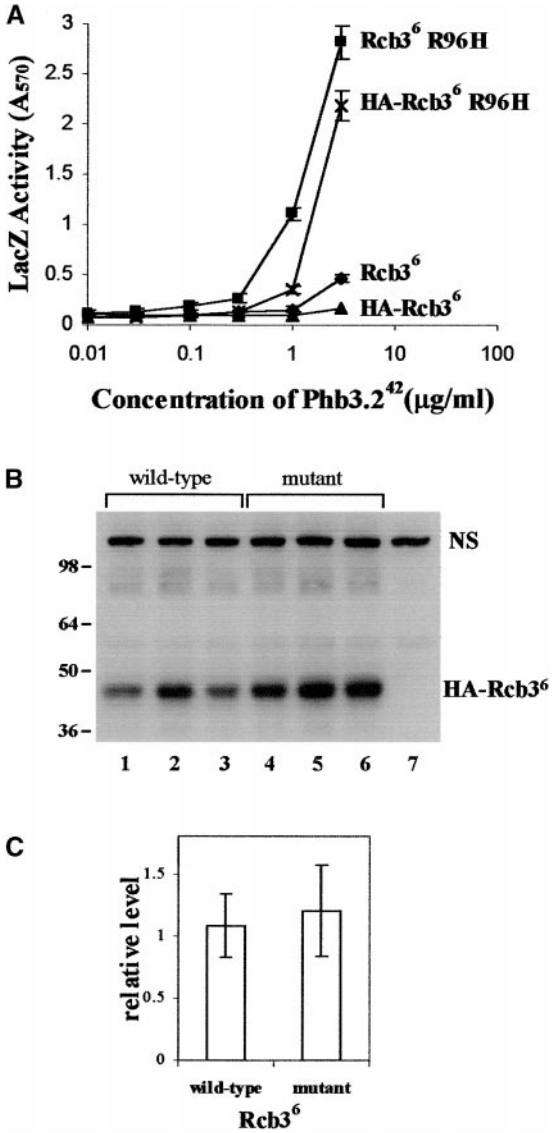

FIGURE 3.—Rcb3^6 and Rcb3^6 R96H respond to synthetic Phb3.2^{42} pheromone. (A) Yeast cells (strain MMY23) expressing either HA-epitope-tagged or untagged versions of Rcb3^6 or Rcb3^6 R96H receptor were incubated with Phb3.2^{42} peptide. *FUS1-lacZ* activities were measured with the substrate CPRG, and conversion to the red product was measured after 48 hr by absorbance at 570 nm. Data are mean ± SD of four independent transformants. (B) Western blot performed on whole-cell extracts from MMY23 cells expressing either HA-Rcb3^6 (lanes 1–3) or HA-Rcb3^6 R96H (lanes 4–6). Extracts were prepared from three independent transformants in each case. Lane 7 contained an extract from cells lacking receptor. The blot was probed with anti-HA mouse monoclonal antibody. Positions of molecular weight markers (kD) are shown. (C) Quantification of Rcb3^6 band in B, after densitometry. Data show relative levels (mean ± SD) after normalization against the nonspecific band (NS) that migrates at >100 kD.

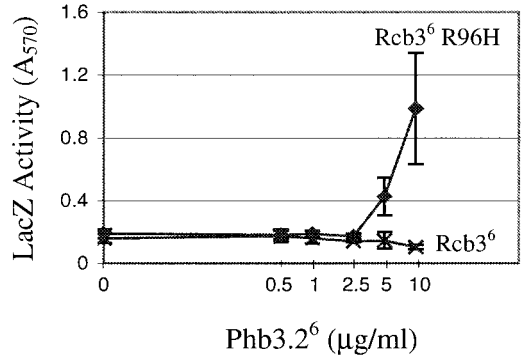

FIGURE 4.—Rcb3^6 R96H responds to a pheromone encoded at the same *B6* locus. Yeast cells (strain MMY23) expressing either Rcb3^6 or Rcb3^6 R96H were incubated with increasing concentrations of Phb3.2^6 peptide. *FUS1-lacZ* activities were measured using the CPRG assay.

These data suggest that the R96H mutation does not affect total cellular levels of receptor protein.

The R96H mutation is unlikely to affect G-protein specificity, as the profile of G-protein coupling specificities of Rcb3^6 and Rcb3^6 R96H among the various chimeric G_α subunits tested was identical (data not shown). This does not preclude the possibility that the mutation might cause increased efficiency of G-protein activation or that the specificity for the particular G_α subunits present in *C. cinereus* (which are not yet identified) might be affected. However, the only clear phenotype observed in experiments with the compatible pheromone was an enhanced ligand response. Since this does not explain the basis of self-compatibility in *C. cinereus*, which occurs in the absence of such a compatible pheromone, we tested whether Rcb3^6 R96H receptor could be activated by one of the normally incompatible pheromones present in the endogenous *B6* cellular environment in *C. cinereus*.

The pheromones encoded by the six genes at the *B6* locus are highly diverged. However, within groups and between alleles there is generally a much greater degree of conservation, both of receptors and pheromones. The sequence of Phb3.2^6 differs at only four amino acid positions from that of Phb3.2^{42}, the pheromone that activates Rcb3^6 (see Figure 1). Moreover, the allelic Rcb3^6 and Rcb3^{42} receptors that discriminate between these two pheromones are also highly conserved with 78% identity in amino acid sequence (HALSALL *et al.* 2000). The most likely candidate pheromone to activate Rcb3^6 R96H to cause self-compatibility was, therefore, Phb3.2^6. Phb3.2^6 was synthesized as a 14-mer lipopeptide ERRTHGGNGLTFWC-SFar(OMe) and tested for its ability to activate *FUS1-lacZ* in yeast cells expressing Rcb3^6 or Rcb3^6 R96H. As expected, synthetic Phb3.2^6 failed to activate wild-type Rcb3^6. However, Phb3.2^6 caused significant activation of the mutant Rcb3^6 R96H (Figure 4). This result offered a clear explanation for the self-compatible phenotype conferred by the R96H mutation.

Since the mutation in Rcb3^6 results in altered specificity, we next probed to what extent this relaxation in

specificity could lead to activation by other synthetic pheromones. We tested the effect of two pheromones from the group 2 family, one encoded by the *B6* locus and the other by the *B42* locus (Phb2.2^{6} and Phb2.2^{42}, respectively; see Figure 1). Neither of these pheromones activated either wild-type or mutant Rcb3^{6} receptor (Figure 5A). The Rcb3^{6} R96H mutation, therefore, does not cause promiscuous activation of this receptor by pheromones; the response to Phb3.2^{6} is ligand specific.

The *B* locus encodes structurally distinct subfamilies of signaling molecules: The intracellular end of TMD III and beginning of intracellular loop 2, where the Rcb3^{6} R96H mutation is located, is reasonably well conserved in the group 2 receptor encoded at the *B6* locus, Rcb2^{6} (Figure 5A). Since Rcb2^{6} also couples in yeast, we tested whether the analogous mutation in Rcb2^{6} conferred a similar self-compatible phenotype. The Rcb2^{6} R95H mutant was created by site-directed mutagenesis and tested for function by transformation into both *B6* and *B42* host strains of *C. cinereus*. In the *B42* background, the Rcb2^{6} R95H activated *B*-regulated development in the mating assay, in common with wild-type Rcb2^{6}. In the *B6* host, Rcb2^{6} R95H failed to confer a self-compatible phenotype and was thus indistinguishable from wild type (data not shown).

To confirm these results, we tested the activity of synthetic pheromones in activating Rcb2^{6} R95H expressed in yeast. The mutation had no significant effect on activation of Rcb2^{6} by the compatible *B42* pheromone Phb2.2^{42} either at a maximal concentration of pheromone (Figure 5C) or at submaximal concentrations (data not shown). Also, the mutation did not confer any ability to respond to the self *B6* pheromone, Phb2.2^{6} (Figure 5C). The effect of the amino acid substitution at this position in the receptor protein is thus specific to Rcb3^{6}, indicating that the structural similarity of this region is not sufficient in itself to cause predictable changes in receptor function.

The genetic studies of O'Shea *et al.* (1998) and Halsall *et al.* (2000) clearly indicate that the three groups of genes at the *B6* and *B42* loci are paralogous and encode functionally discrete subfamilies of signaling molecules. The yeast assay has permitted us to confirm this directly for the group 2 and group 3 genes. We have shown above that neither the wild-type nor the mutant Rcb3^{6} receptor could be activated by a group 2 pheromone. Figure 5C shows that the group 2 receptor, Rcb2^{6}, could not be activated by a group 3 pheromone. We also tested the constitutively active Rcb2^{6} Q229P mutant receptor and found that it too did not respond to a group 3 pheromone.

F67W substitution in the pheromone Phb3.2^{6} causes self-compatibility: The self-compatible *B* mutations selected in *C. cinereus* both arose in receptor genes. From comparison of the pheromone sequences it seemed likely that pheromone mutations might result in the same phenotype. As described above, the predicted

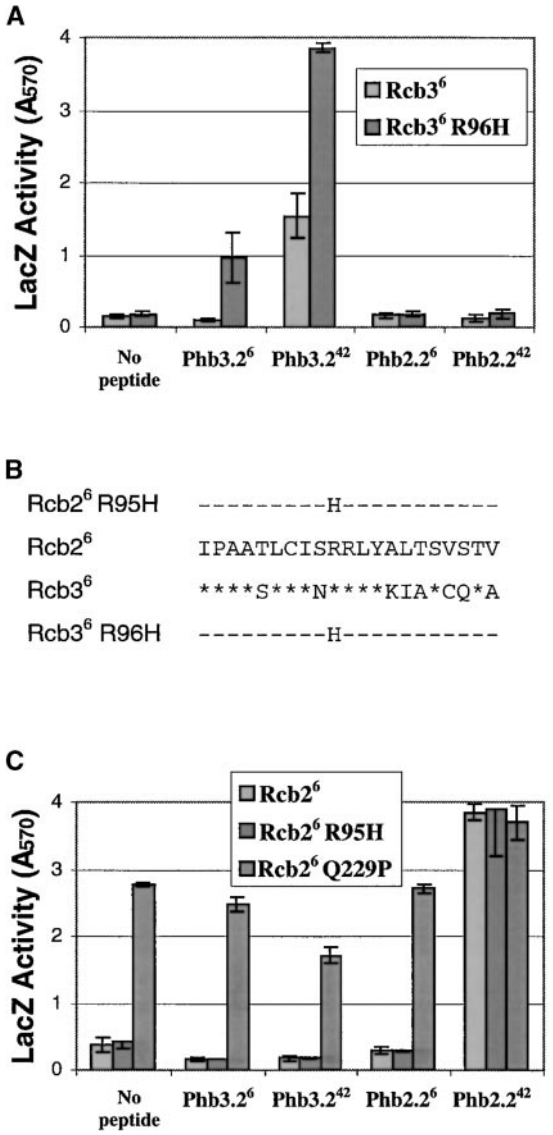

Figure 5.—Pheromone activities at wild-type and mutant Rcb2^{6} and Rcb3^{6} receptors. (A) Responses of wild-type Rcb3^{6} and mutant Rcb3^{6} R96H to the four available synthetic pheromones, measured in MMY23 cells using the CPRG assay of *FUS1-lacZ* activity. Cells were incubated with 10 μg/ml of each synthetic peptide except Phb3.2^{42}, which was used at 3.5 μg/ml, due to toxicity at higher concentrations. (B) Comparison of the amino acid sequences of the Rcb2^{6} and Rcb3^{6} receptors at the junction of TMD III and intracellular loop 2, showing the analogous R95H/R96H substitutions. Asterisk indicates an identical amino acid between Rcb2^{6} and Rcb3^{6}. (C) Responses of wild-type Rcb2^{6} and mutants Rcb2^{6} R95H and Rcb2^{6} Q229P to the four synthetic pheromones. This assay was performed in yeast strain MMY16, which contains the Gpa1/$G_{\alpha 16}$ chimeric G-protein that supports efficient coupling of Rcb2^{6}. Concentrations of synthetic peptides were as above.

amino acid sequences of mature Phb3.2^{6} and Phb3.2^{42} pheromones differ at only four positions (Figure 6A). To identify which of these amino acids determines specificity, we introduced mutations into the Phb3.2^{6} pheromone precursor gene at each of the four positions in

A

Phb3.2^{6}	ERRTHGGNGLTFWC
Phb3.2^{42}	****Q**G***WF*

B

		Activity in host	
		B42	*B6*
Phb3.2^{6}	ERRTHGGNGLTFWC	+	−
Phb3.2^{6} H60Q	****Q*********	+	−
Phb3.2^{6} N63G	*******G******	+	−
Phb3.2^{6} F67W	**********W**	+	+
Phb3.2^{6} W68F	**********F*	+	−
Phb3.2^{6} F67W/W68F	**********WF*	−	+

Figure 6.—A single amino acid substitution in a pheromone causes mating self-compatibility. (A) Comparison of the predicted mature peptide sequences of pheromones Phb3.2^{6} and Phb3.2^{42}. (B) Amino acid substitutions created in Phb3.2^{6} and their effects on mating in *B6* and *B42 C. cinereus* host strains. Phb3.2^{6} F67W stimulates both *B6* and *B42* receptors and confers self-compatibility. An additional substitution in Phb3.2^{6}, W68F, alters specificity further in that the double mutant does not activate the *B42* strain but does activate the *B6* strain.

turn, changing the amino acid to that in Phb3.2^{42} (Figure 6B). The mutant genes were introduced into *B6* and *B42* host strains of *C. cinereus* to determine their ability to activate *B*-regulated development. Three of the mutant Phb3.2^{6} pheromones (H60Q, N63G, and W68F) behaved like the wild type and were active only in the *B42* background. In contrast, F67W was active in the *B42* background but could also confer self-compatibility on the *B6* strain. This altered specificity suggests that the Phb3.2^{6} F67W mutant is able to activate both Rcb3^{6} and Rcb3^{42} receptors and illustrates how single amino acid changes in either pheromone or receptor can result in self-compatibility due to a relaxation of the normal specificity of pheromone/receptor recognition. It is unlikely that the previous screen (Haylock *et al.* 1980) would have yielded the Phb3.2^{6} F67W mutation as this amino acid substitution involves a double nucleotide change.

To examine the significance of the Phe Trp/Trp Phe (FW/WF) motif at the C termini of Phb3.2^{6} and Phb3.2^{42}, we created the double-mutant Phb3.2^{6} F67W/W68F in which both amino acids in the *B6* pheromone were exchanged to those of Phb3.2^{42}. The Phb3.2^{6} F67W/W68F double mutant conferred self-compatibility in that it activated *B*-regulated development in the *B6 C. cinereus* host. However, it had barely detectable activity in the *B42* host, suggesting it had greatly reduced ability to activate the normally compatible Rcb3^{42} receptor (Figure 6B). It is remarkable that while a single amino acid change can generate a self-compatible pheromone, the double replacement reverses the specificity almost entirely. These two amino acids together are thus critical in determining the specificity of the naturally occurring pheromones with respect to the *B6* and *B42* receptors.

DISCUSSION

Mutations that confer *B* mating self-compatibility are difficult to generate in *C. cinereus*. They cause activation of the pheromone response pathway without the need for mating and lead to constitutive expression of *B*-regulated sexual development. We now demonstrate that there are at least three mechanisms whereby this can occur. The three mutations described by Haylock *et al.* (1980) in the *B6* locus all give rise to mutant receptors. In two cases, identical amino acid substitutions (Olesnicky *et al.* 1999) generated a constitutively activated receptor that no longer requires pheromone stimulation. In the third case, which we describe here, a single amino acid has caused a change in pheromone specificity such that the receptor is activated by a normally incompatible pheromone encoded at the same *B* locus. We generated a third class of mutant by introducing single amino acid substitutions in a pheromone. This altered its receptor specificity so that it could activate a normally incompatible receptor encoded at the same *B* locus. Although the mutation was engineered, recent studies on *S. commune* confirm that this class of pheromone mutation can also arise *in vivo* (Fowler *et al.* 1998).

Genetic data led us to propose that the genes within the *B* locus of *C. cinereus* encode three functionally discrete families of signaling molecules. *B6* and *B42* were found to share alleles of the group 1 genes but have different alleles of the group 2 and group 3 genes (Halsall *et al.* 2000). Transformation data showed that the group 2 pheromone Phb2.2^{42} and the group 3 pheromone Phb3.2^{42} both activate a compatible receptor in a *B6* host. Here we confirm, using the yeast assay, that Phb2.2^{42} specifically activates the group 2 receptor and Phb3.2^{42} specifically activates the group 3 receptor. Our mutation data show that a single amino acid change in either the group 3 pheromone or the receptor is sufficient to change the specificity. This emphasizes the fact that each family of signaling molecules encoded by the *B* genes has evolved through very subtle changes in its members. We noted that the mutant Rcb3^{6} R96H responds more strongly to the compatible ligand Phb3.2^{42} than wild-type Rcb3^{6}. Transformation experiments in *C. cinereus* show that a given pheromone is not equally effective in promoting a compatible response in different genetic backgrounds (Halsall *et al.* 2000). Thus the wide spectrum of activity demanded of each pheromone and receptor may have been achieved by sacrificing full efficiency in some combinations.

The receptor mutant that we describe in this article, Rcb3[6] R96H, has three key features that make it interesting: it does not appear to be constitutively active, it has an enhanced response to a compatible ligand, and it responds erroneously to a normally incompatible ligand. This latter phenotype has been described in mutants of yeast pheromone receptor Ste2p. These mutants could be activated by certain pheromone analogues that bound but did not activate the wild-type receptor (Marsh 1992; Abel *et al.* 1998). However, the mutations occurred in the receptor hydrophobic core of Ste2p at positions that feasibly could contact ligands directly. In contrast, the location of R96 in the intracellular loop makes it unlikely that the residue at this position contacts ligand directly, and the effect on ligand specificity is remarkable. There is clearly a close connection between the gross structural features of a receptor that influence ligand binding, constitutive activity, and G-protein activation.

Movements of TMDs III and VI play a key role in the activation of GPCRs (Yu *et al.* 1995; Farrens *et al.* 1996; Sheikh *et al.* 1996; Gether *et al.* 1997). However, the precise mechanism of receptor activation remains unclear. Growing evidence implicates the intracellular end of TMD III as having a critical role at the interface between receptor conformation and G-protein activation. Many mammalian GPCRs in the rhodopsin-like subfamily (family A) have a characteristic Asp Arg Tyr (DRY) motif at a position corresponding to the *C. cinereus* Rcb3[6] mutation and changes in this motif can have various effects on either ligand binding or G-protein coupling. For example, mutagenesis of the α_{1B} adrenergic receptor has shown that mutation of D142 of the DRY motif to any other amino acid constitutively activates the receptor (Scheer *et al.* 1996; Mhaouty-Kodja *et al.* 1999). Also, an R to H mutation in the DRY sequence of the β_2-adrenergic receptor causes an increase in ligand binding affinity (Seibold *et al.* 1998), and an S140Y mutation in the corresponding DRS motif of the GnRH receptor caused a 100% increase in ligand binding and internalization (Arora *et al.* 1995, 1997). Family E GPCRs, such as the *C. cinereus* receptors, have little sequence homology to family A and do not contain a DRY motif. However, the second intracellular loop is highly conserved among the fungal pheromone receptors. R96 of Rcb3[6] forms part of an Arg Leu Tyr (RLY) motif (Figure 5B) and lies in what may be the equivalent position to the aspartate of the mammalian DRY sequences. The isolation of the R96H mutation in a random screen highlights the importance of the TMDIII/intracellular loop 2 region across all families of GPCRs. Surprisingly, a mutation equivalent to Rcb3[6] R96H engineered into the group 2 receptor Rcb2[6] neither produced a self-compatible phenotype nor altered the ligand specificity. Thus, the R to H mutation in this position does not have a generic effect on receptor activation and illustrates that it is not possible to predict the effect of a given mutation on receptor function.

C. cinereus and *S. commune* are useful models in which receptor and pheromone functions can be measured using a genuine biological phenotype. For both species, a yeast assay has been developed for studying isolated receptor-ligand interactions (Fowler *et al.* 1999; Olesnicky *et al.* 1999). Here we show that, using a yeast assay, it is possible to distinguish the effect of mutations on ligand-dependent receptor activation from those that affect the intrinsic receptor activity in the absence of ligand and that give identical phenotypes in the biological assay. The combination of these systems promises to give further insight into receptor behavior that can undoubtedly be applied to other eukaryotic systems.

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