MOD-D, a Gα Subunit of the Fungus *Podospora anserina*, Is Involved in Both Regulation of Development and Vegetative Incompatibility

Gabriel Loubradou, Joël Bégueret and Béatrice Turcq

Laboratoire de Génétique Moléculaire des Champignons Filamenteux, Institut de Biochimie et de Génétique Cellulaires, CNRS UPR 9026, 33077 Bordeaux, France

Manuscript received June 5, 1998
Accepted for publication March 10, 1999

ABSTRACT

Cell death via vegetative incompatibility is widespread in fungi but molecular mechanism and biological function of the process are poorly understood. One way to investigate this phenomenon was to study genes named *mod* that modified incompatibility reaction. In this study, we cloned the *mod-D* gene that encodes a Gα protein. The *mod-D* mutant strains present developmental defects. Previously, we showed that the *mod-E* gene encodes an HSP90. The *mod-E1* mutation suppresses both vegetative incompatibility and developmental defects due to the *mod-D* mutation. Moreover, we isolated the *PaAC* gene, which encodes an adenylate cyclase, as a partial suppressor of the *mod-D1* mutation. Our previous results showed that the molecular mechanisms involved in vegetative incompatibility and developmental pathways are connected, suggesting that vegetative incompatibility may result from disorders in some developmental steps. Our new result corroborates the involvement of *mod* genes in signal transduction pathways. As expected, we showed that an increase in the cAMP level is able to suppress the defects in vegetative growth due to the *mod-D1* mutation. However, cAMP increase has no influence on the suppressor effect of the *mod-D1* mutation on vegetative incompatibility, suggesting that this suppressor effect is independent of the cAMP pathway.

In filamentous fungi, formation of heterokaryotic cells by hyphal fusion is controlled through a mechanism of somatic or vegetative incompatibility (for reviews see Glass and Kuldau 1992; Bégueret *et al.* 1994). Vegetative incompatibility is triggered by genetic differences at specific loci named *het* loci. Some *het* genes have been isolated and characterized in *Podospora anserina* (Turcq *et al.* 1990; Saupe *et al.* 1994, 1995) and *Neurospora crassa* (Glass *et al.* 1988, 1990; Staben and Yanofsky 1990; Saupe *et al.* 1996). However, information obtained from the molecular data did not suggest any common function among *het* genes. Vegetative incompatibility triggered by the coexpression of *het* genes is still not understood.

An alternative approach to understanding mechanisms that regulate vegetative incompatibility is to study mutations that interfere with this phenomenon. Such mutations have been isolated in *N. crassa* (Newmeyer 1970; Arganoza *et al.* 1994; Vellani *et al.* 1994) and in *P. anserina* (Belcour and Bernet 1969; Bernet 1971; Labarère and Bernet 1977, 1979a; Durrens 1982, 1984). In *P. anserina*, these mutations occur in *mod* genes (modifiers of incompatibility reaction). Mutations in *mod* genes induce alterations in differentiation steps. In addition to being involved in vegetative incompatibility, *mod* and *het* genes may control some steps of the life cycle of the fungus (Boucherie *et al.* 1976).

In *P. anserina*, coexpression of incompatible *het* genes leads to a growth arrest and a cell death by lytic reaction. Mutant strains that display only the lytic reaction were obtained. Using two different screening procedures, three *mod-D* mutations were selected via the ability to restore growth of these mutant strains. The *mod-D1* mutant was selected from a *het-C het-E mod-A1 mod-C1* genetic background in which *het-C* and *het-E* are incompatible (Labarère and Bernet 1979a). The *mod-D2* and *mod-D3* mutants were selected from the self-lytic *mod-A1 mod-B11* strain (Durrens *et al.* 1979). None of these *mod-D* mutations is able, by itself, to suppress cell lysis and growth arrest due to vegetative incompatibility. Moreover, the three *mod-D* mutants are altered in the differentiation of secondary ramifications (*i.e.*, distorted and slow-growing hyphae), aerial hyphae, and protoperithecia (Durrens *et al.* 1979; Labarère and Bernet 1979a,b). They also display a decrease in the renewal of growth from stationary cells and a defect in spore germination (Durrens *et al.* 1979; Labarère and Bernet 1979a,b). The *mod-D2* strain exhibits the most altered phenotype and it is also deficient for pigmentation. P. Durrens and J. Bernet proposed that *mod-D* controls the escape from the stationary state. This state would be a prerequisite for the formation of differentiated structures (Durrens and Bernet 1982).

In a previous attempt to clone the *mod-D* gene by

Corresponding author: Béatrice Turcq, Laboratoire de Génétique Moléculaire des Champignons Filamenteux, Institut de Biochimie et de Génétique Cellulaires, CNRS UPR 9026, 1 rue Camille Saint-Saëns, 33077 Bordeaux cedex, France.
E-mail: beatrice.turcq@ibgc.u-bordeaux2.fr

complementation of the *mod-D1* mutation, *PaAC*, a gene encoding an adenylate cyclase, was isolated. An ectopic copy of *PaAC* is able to complement the disorders in vegetative growth of the *mod-D1* mutant but not the defect in spore germination (Loubradou *et al.* 1996). In a parallel approach, we have identified a gene, *mod-E*, whose mutation suppressed developmental defects associated with *mod-D2* mutation (Durrens 1982). The *mod-E1* mutation is epistatic to the *mod-D2* mutation for all developmental disorders (Durrens 1982). Moreover, the *mod-E1* mutation can partly restore the growth of a self-incompatible *het-R het-V* strain (Loubradou *et al.* 1997). These results illustrate the tight connection existing between different pathways involved in development and in vegetative incompatibility. The *mod-E* gene encodes an HSP90 (Loubradou *et al.* 1997). Proteins of this family are known to be involved in signal transduction pathways in interaction with nuclear receptors or protein kinases (for review see Csermely *et al.* 1998).

Characterization of the *mod-D* gene is expected to provide additional information about pathways involving *PaAC* and *mod-E*. We report here the cloning of the *mod-D* gene and we show that it encodes an α subunit of a heterotrimeric G protein. The sites mutated in the three *mod-D* mutants were characterized. To further investigate the functional alteration caused by these mutations, a putative constitutively active allele was created by site-directed mutagenesis. The involvement of cAMP during the vegetative growth was demonstrated for *mod-D1* and *mod-D3* mutants. No effect was observed on the *mod-D2* mutant strain.

To define precisely the function of *mod-D* in vegetative incompatibility, and also to test cAMP involvement in vegetative incompatibility, we investigated the ability of *mod-D* mutations to restore the growth of an incompatible strain in different concentrations of cAMP. The *mod-D1* and *mod-D2* mutations are able to partially restore the growth of this strain. An increase in the cAMP concentration does not interfere with this restoration of growth. It shows that the suppressor effect of *mod-D* mutations is not due to a decrease in cAMP concentration for this phenotype.

MATERIALS AND METHODS

***P. anserina* strains, growth conditions, and transformation:** *P. anserina* is a heterothallic ascomycete. Life cycle and general methods for genetic analysis have been described (Rizet and Engelman 1949; Esser 1974). The *het-C het-E* strain is an autolytic strain due to the coexpression of the two nonallelic incompatible genes *het-C* and *het-E*. The *mod-A1* mutation has been isolated by its ability to modify the incompatibility reaction (Belcour and Bernet 1969).

D0 and synthetic medium have been previously described (Loubradou *et al.* 1997). Theophylline (1,3-dimethylxanthine; Sigma, St. Louis) and dibutyril cAMP (N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate; Sigma) were added to these media when indicated.

Protoplasts were prepared and transformed as described in Barreau and Bergès (1989). The pMOcosX vector containing the bacterial hygromycin resistance gene *hph* was used as a selectable marker (Orbach *et al.* 1991), and transformants were screened on hygromycin B at 100 μg/ml. The library construction has been described elsewhere (Loubradou *et al.* 1996).

Cloning of the *mod-D* mutant alleles and *mod-D1* cDNA: Genomic DNA of the *mod-D* mutant strains was prepared using the rapid Petri dish-grown mycelia method (Lecellier and Silar 1994). PCR amplification of DNA (Saiki *et al.* 1988) was achieved in the buffer III described in Ponce and Micol (1992), using 100 ng of each primer and 20 ng genomic DNA in a 50-μl mixture. Two pairs of primers were used: Mut-D3 5′AGGGAAGGAGCGACACAATAG3′ (861-881) and Mut-D4 5′GGTTTTTGAACACGGTGGGTC3′ (1775-1755), or Mut-D5 5′CTGCAGCTAGATTCCCCGATA3′ (3057-3037) and Mut-D6 5′AGCGGCAAGTCAACTATTGTG3′ (1676-1696). After 12 min at 95°, DNA was amplified for 35 cycles in a Perkin Elmer-Cetus (Norwalk, CT) thermocycler. The cycling parameters were as follows: 95° for 30 sec, 56° for 2 min for Mut-D3 and Mut-D4 primers, or 53° for 2 min for Mut-D5 and Mut-D6 primers, and 72° for 2 min. Synthesis of the *mod-D1* cDNA and PCR amplification was performed using the Access RT-PCR system kit (Promega, Madison, WI). Total RNA was prepared as described previously (Turcq and Bégueret 1987). The primers were Mut-D6 and RT1 5′CAACTCTGGAG GATGCATGAG3′ (2857-2837). The cycling parameters were as follows: 94° for 30 sec, 52° for 1 min, and 72° for 2 min.

Plasmids, DNA sequencing, and site-directed mutagenesis: All plasmids were constructed using standard methods (Sambrook *et al.* 1989) and were propagated in *Escherichia coli* XL1blue. The *mod-D* gene was cloned into the pBluescript SK^+ vector (Stratagene, La Jolla, CA). Subclones for sequencing were produced using restriction sites. Nucleotide sequencing was performed using the dideoxynucleotide chain termination method (Sanger *et al.* 1977) with the Sequenase version 2.0 reagent kit (U.S. Biochemical Corp., Cleveland) and [α-^{35}S]dATP as the label. Initially, pBluescript commercial primers were used and then the sequence was completed with 17-mer synthetic internal primers derived from the available sequence. The Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) was used for site-directed mutagenesis with the following oligonucleotides: ARG201 5′GCTTCGG GCGTGCACGAAGACCAC3′ (2125-2148) and MutSal 5′CAC GGTCGGTAATCTGGTCCTCC3′ (884-906).

Nucleotide sequence accession number: The nucleotide sequence data reported in Figure 1 will appear in the GenBank/EMBL/DDBJ nucleotide sequence database under accession no. AF038122.

RESULTS

Cloning of the *mod-D* gene: In a previous attempt to isolate the *mod-D* gene, *PaAC*, a gene encoding an adenylate cyclase, was cloned as a partial physiological suppressor of the *mod-D1* mutation (Loubradou *et al.* 1996). An additional ectopic copy of the *PaAC* gene is able to complement vegetative growth disorders due to the *mod-D1* mutation but the spore germination defect caused by the mutation cannot be restored. To avoid reisolation of the *PaAC* gene, a two-step screening procedure was used to clone *mod-D*. Transformants were first screened on synthetic medium for the restoration of a wild-type phenotype of the mycelium, and the strains were afterwards examined for the restoration of spore

germination. Sixteen pools of 192 clones each from a wild-type genomic DNA library constructed in the pMOcosX cosmid (Loubradou *et al.* 1996) were used to transform protoplasts of the *mod-D1* strain. One strain out of the 2300 transformant strains displayed a wild-type phenotype on synthetic medium and a wild-type spore germination rate. The corresponding cosmid was isolated by SIB selection (Akins and Lambowitz 1985). Genetic analysis of three transformants showed that for two of them the cosmid was integrated at the *mod-D* locus. The complementation was likely due to rescue of *mod-D* gene function and not to the cloning or creation of an unlinked suppressor. This result was confirmed by the characterization of the sites mutated in the *mod-D* mutant alleles (see below).

MOD-D is an α subunit of a heterotrimeric G protein: Ectopic integration of a 3057-bp *Pst*I-*Kpn*I fragment fully complements the defects due to the *mod-D1* mutation. The sequence of the fragment encompassing *mod-D* revealed the presence of an open reading frame (ORF) of 1374 bp interrupted by five putative introns according to consensus sequences for filamentous fungi splicing sites (Ballance 1991; Bruchez *et al.* 1993; Figure 1). The gene has the ability to encode a 354-amino acid (aa) polypeptide. The comparison between the *mod-D*-encoded polypeptide, designated MOD-D, and the protein sequences in the GenBank database indicated strong similarities with all α subunits of heterotrimeric G proteins. For example, 87.5, 87, 69, 69, and 65% identity were observed with magA from *Magnaporthe grisea* (Liu and Dean 1997), CPG-2 from *Cryphonectria parasitica* (Choi *et al.* 1995), Gpa3 from *Ustilago maydis* (Regenfelder *et al.* 1997), Fil1 from *Ustilago hordei* (Lichter and Mills 1997), and Gpa1 from *Cryptococcus neoformans* (Tolkacheva *et al.* 1994), respectively. The consensus sequences for the GTP-binding site, sequences G-1, G-2, G-3, G-4, and G-5 (Bourne *et al.* 1991), are all present in the MOD-D sequence (Figure 1). These results show that MOD-D encodes an α subunit of a heterotrimeric G protein.

Different families have been defined for Gα proteins from higher eucaryotes (Simon *et al.* 1991). In fungi, such classification has not been established before but a previous phylogenetic analysis indicates that several subgroups could be identified (Regenfelder *et al.* 1997). We did a similar analysis including MOD-D. This was performed by using the Phylogeny Inference package version 5.3c. (Felsenstein 1993; Figure 2). The results show that MOD-D, magA from *M. grisea*, CPG-2 from *C. parasitica*, Gpa1 from *C. neoformans*, Fil1 from *U. hordei*, and Gpa3 from *U. maydis* may define a family of Gα proteins in fungi. The yeast Gpa2 proteins from *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* could be related to this family since they belong to the same branch of the phylogenetic tree. Sequence comparisons show that MOD-D from *P. anserina*, magA from *M. grisea*, CPG-2 from *C. parasitica*, Gpa1 from *C. neoformans*, Fil1 from *U. hordei*, and Gpa3 from *U. maydis* share a very high level of identity in their C-terminal half. There is 84% identity among MOD-D, CPG-2, magA, Gpa3, Fil1, and Gpa1 within the last 56 amino acids, but only 40% identity within the same region if Gpa1 or Gpa2 from *U. maydis* is included in the comparison (Regenfelder *et al.* 1997; Figure 2). This strong sequence conservation is therefore specific for this particular family and not observed for all fungal Gα proteins.

Identification of mutations in *mod-D* mutant alleles: Relations between structure and function have been extensively studied for Gα proteins (for reviews see Conklin and Bourne 1993; Neer 1995). To obtain information on the functional defect of the *mod-D* mutant proteins, we identified the mutations present in *mod-D* mutant alleles. The three *mod-D* mutant genes were isolated from genomic DNA by PCR. Two clones from distinct PCR reactions were sequenced for each mutant and a unique mutation was identified for each allele (Figure 1). In *mod-D1* and *mod-D3*, the mutation is located in the 3′ acceptor site of the second and fourth introns, respectively. In the *mod-D2* allele, the stop codon is mutated. The next stop codon in the sequence is located 62 codons downstream from the stop codon of the wild-type ORF. Therefore, the consequence of the *mod-D2* mutation should be a protein with an additional C-terminal sequence of 62 aa. The consequences of *mod-D1* and *mod-D3* mutations on the function of the protein are difficult to predict because they depend on the splicing positions in the mutant mRNA. As *mod-D1* and *mod-D3* mutations induce similar phenotypes, the mutant proteins presumably share similar functional defects. We decided to analyze only the splicing of *mod-D1* mRNA.

MOD-D1 could be unable to activate its effector: cDNA was synthesized from *mod-D1* mRNA and amplified by PCR using internal specific primers. These primers amplify a fragment corresponding to positions 1676-2857 in the genomic DNA (Figure 1). The fragment includes the last four introns of the gene. Two fragments with equal intensity were visualized after migration of the PCR products on agarose gel with ethidium bromide staining. Thus the amount of the two mRNA populations is equivalent. These fragments were cloned. Only one clone with the large fragment and six clones with the short fragment were partially sequenced to determine splicing junctions for the second and the fifth introns. In all cases, the fifth intron was absent, showing that all sequenced fragments were derived from *mod-D1* cDNA and not from genomic DNA. The sequence of the large fragment showed that the second intron is still present, whereas in the short fragment, the second intron is absent but the splicing occurred at different positions. Five of the six clones were spliced using the first G downstream from the mutated position, whereas the last one was spliced using the first AG following the

```
   1 GGTACCCGTTGACAGCTTCATTGCGGTGGAGCGTGTGTTGGGCCGGTCCAACACACACCCGGTGGAAGTACAATGGCCTC
  81 TCAACACGTCCAACAAGGCTTGGAACTCACGACGCTGTACCTGGATGGATGGGCACGTCGAGCTGGAGCTCCCATGCTGG
 161 ATCTCGCACCCACTTGTCACGGCATCCATTTCGTGATTCTGTGGGGCCAGATCTGCGTTACCAGCAAGACCCGTTCCCAT
 241 CACCAGCCGGTGGGCCACGGAGCACGGCCGGCAAGGCTCTCCTGTGGTGAAGAGACGGTGGCCAATGCGGCCCCTGGAAC
 321 ACCAAGACTCGATACCTTCTCTTTGCAGTGCGGTGTGTCGCATCTCGAGGTATCTCATGTTTGGCCTGTCAGAAGAGCCC
 401 GGGACCCCATGTTTTCCCCCCAGGCGCTGTCTAATTGCCCTGGGGAGCTGCCGCCTGGGCTACAGTGCCGTAAAATGCGC
 481 CAGCGTGGTGAAGCGAAAGAGGGCGAGCAAAAACTCCAGTTCCACCACCTCAAGGCTCCCCGGTCTGTGCTCCGTGCTCC
 561 CGTCCAGGTCCTCTGCCAGCTTCCACTTCCGTTTCCAGGTGCTTGCATTTCCAGTTTTCCTTCTGTCATCACCATCTCAG
 641 GTGGTTTCGACAAAATAACGAGGATATCAGGCATTGTATCGCCCTTTGCTATTTCAATCCATCTCGGCCTGTCCCCACCG
 721 ACACCAGTCACGCTTGAGTAGTGCGTGATAAGGACAGGAGCAAGTGCAAGTGACGGCTTATTAGTCCCGTTCTCCCGCAA
 801 CGCAACGGTCAACGGTTTGACAGCGCTCCGACTGAAAGCGAAACCAAAACAACACAACCAAGGGAAGGAGCGACACAATA
 881 GTTCACGGTCGGTCGACTGGTCCTCCCAAACAACACCAATTCGCCTTTTATCGCCAGCCTGACCTTGTTGCCGAATCAGA
 961 CCCTCGACTTGCTTGAGCTTCGAGGCTCCATTGAGGCCCCATACAAAAGCTGGGGAGGGGGTCCGAGGCGGGTCTTTGTG
1041 TTGTTGCATCGCTACCTCTTCGACTCTCTCAATCGGGATCCAAGGCCACCTCTCTCCGCCCGGCCAAGGCAGTTCTACGA
1121 CCTGCACAAGGCAACCTCGAACCCAACAGAGACGGCTTCTCTCTGCACGGCATTTCGGCATTCCGAACCCTCACCCTCAC
1201 CGACCAACACTATCAATCCATCCATCACCCAGCACCTCGCAGCCATCGCAAACAGCCACGACCGCCCGCGCCTATGGGTT
1281 TCTAGGGAAAATCCAAGGACAAGCGAGCGCCCACGTCGCCAGCGGCAGCAACCCGCCGTCGCGCCTTCTCAGCTGCTGCC
1361 GCCGCCGACACTGCCGCGGCCAGCCTCTCTACTGCCAGCCGATTGTCGCCCGCGACCTTATCCAGCACTCGCAACGACCG

1441 TCCCCGGAGGTTCATGGTCACAATGGGGTCGTGCATGAGCACGAGCGGGGAGGAATCGGAACAGCGGAAGAGGAGCAACA
   1                     M  G  S  C  M  S  T  S  G  E  E  S  E  Q  R  K  R  S  N  K

1521 AGATTGACAGAGATCTGGAGGAAGATTCAAAACGGCTGCGCCGAGAATGCAAGATTCTACTGCTTGGTACGTCAATTGTC
  21   I  D  R  D  L  E  E  D  S  K  R  L  R  R  E  C  K  I  L  L  L  G

1601 TGCTTCGCCGCGCGAGCCGCCAAGTCGCGACTTTCCATTTGCTAACCACTTGTTACACACGCAGGCTCTGGTGAGAGCGG
  43                                                                  S  G  E  S  G
                                                                      G1

1681 CAAGTCAACTATTGTGAAGCAGATGAAAATCATCCACCTGAAAGGTTATTCGCATGAGGAGCTCAAGAATTACAGACCCA
  48  K  S  T  I  V  K  Q  M  K  I  I  H  L  K  G  Y  S  H  E  E  L  K  N  Y  R  P  T

1761 CCGTGTTCAAAAACCTGCTGGAATGCGCCAAATCGGTCGCCAGCGCCATGCGCCAGTTCGACATCGAGCCAGTACTGGAC
  75   V  F  K  N  L  L  E  C  A  K  S  V  A  S  A  M  R  Q  F  D  I  E  P  V  L  D

1841 GAGAACAAGCGACATCTCGACTTTTTGATTGATTACTCCCTCGATACCAACCCACAGACCATTGACGCAAAGGTCAGCGT
 101 E  N  K  R  H  L  D  F  L  I  D  Y  S  L  D  T  N  P  Q  T  I  D  A  K  V  S  V

1921 GGCGATCCAGTCATTATGGAACGACCCGGCCAAGGAGCAACTCATGGAAAGGCAAACAGAGTTTTACTTGATGGACTCTG
 128  A  I  Q  S  L  W  N  D  P  A  K  E  Q  L  M  E  R  Q  T  E  F  Y  L  M  D  S  A

                                                    mod-D1 mutation A
2001 CGGAATAGTGAGTTTCTTGTGTTTGAACCGAGGGGCACGGACTGGTATCTAACTTCACCGGCAGCTTTTTTGACGAGGCT
 155   E  Y                                                          F  F  D  E  A

2081 GCAAGAATCGCACACAAGGAGTACATCCCCAATGAGATGGATGTGCTTCGGGCGAGAACGAAGACCACAGGTATCTACGA
 162 A  R  I  A  H  K  E  Y  I  P  N  E  M  D  V  L  R  A  R  T  K  T  T  G  I  Y  E
                                                    G2

2161 GACGAGATTCAAGATGGGCCAACTCAGTATTCAGTGAGTTTTCATGCTGCCAATCTTGCCTGGAACAACACTGACAGTTC
 189  T  R  F  K  M  G  Q  L  S  I  H

2241 ACAGCATGTTTGACGTGGGTGGTCAACGCAGCGAACGCAAGAAGTGGATCCACTGTTTTGAGAACGTCACTTCCATCATC
 200      M  F  D  V  G  G  Q  R  S  E  R  K  K  W  I  H  C  F  E  N  V  T  S  I  I
          G3

2321 TTTTGTGTGGCCTTGAGCGAGTACGATCAGGTCTTGCTAGAAGAAAGCAGTCAGGTACGCGATTCGTGGCCCATCAAGTT
 225 F  C  V  A  L  S  E  Y  D  Q  V  L  L  E  E  S  S  Q

                mod-D3 mutation A
2401 ATGTCTCCGTGATGCTAATTGGATCTCTAGAACCGTATGATGGAGTCACTACTACTTTTCGACTCGGTAGTCAACTCACG
 243                              N  R  M  M  E  S  L  L  L  F  D  S  V  V  N  S  R

2481 GTGGTTTATGCGCACGAGTATCATCTTGTTCTTGAATAAGGTTGATATCTTCAAGCAGAAACTGGGCCGATCTCCACTCA
 260  W  F  M  R  T  S  I  I  L  F  L  N  K  V  D  I  F  K  Q  K  L  G  R  S  P  L  S
                                    G4

2561 GCAACTATTTCCCCGATTACTCGGGAGGCAACGACGTCAACAAGGCAGCCAAGTATCTGCTCTGGAGATTCAACCAAGTG
 287   N  Y  F  P  D  Y  S  G  G  N  D  V  N  K  A  A  K  Y  L  L  W  R  F  N  Q  V

2641 AACAGGGCGCATCTAAATCTCTACCCTCAGTAAGTTGTTCTCTGTCCCGCCGTCGTTTTGCTTTTGTTATGATGCTAACG
 313 N  R  A  H  L  N  L  Y  P  H

2721 ATCGATCAGCTTGACACAAGCGACAGATACTAGCAACATCCGTCTGGTTTTTGCTGCGGTTAAGGAGACGATCCTGAACA
 323          L  T  Q  A  T  D  T  S  N  I  R  L  V  F  A  A  V  K  E  T  I  L  N  N
               G5

              mod-D2 mutation A
2801 ACGCGCTGAAGGATTCTGGTATTCTATAGATGCCCCCTCATGCATCCTCCAGAGTTGGAGGCGTACTCCCCGCACGATCG
 347   A  L  K  D  S  G  I  L  ---

2881 GGCGTATTTCAGGACTGCTGTGCCTGGCAGCTGTTGATCCCGAAAGCATCACCCTCCCATCCTACCAAGGAACACAGGCA
2961 CGATTCGATCCGAGGAAAGTCAGCAAATAGCGACACTCGAATCTCTCCTTATTGATTGATAATGTACAGATCCCTCTATC
3041 GGGGAATCTAGCTGCAG
```

Figure 1.—Nucleotide and predicted amino acid sequence of *mod-D*. Consensus regions for splicing sites are italicized (Ballance 1991). Regions conserved among G proteins (G-1, G-2, G-3, G-4, and G-5) are underlined (Bourne *et al.* 1991). Base changes in *mod-D1*, *mod-D2*, and *mod-D3* alleles are in boldface. The arginine residue of the G-2 region underlined by a double line was mutated to cysteine in the *mod-D*^{R180C} allele.

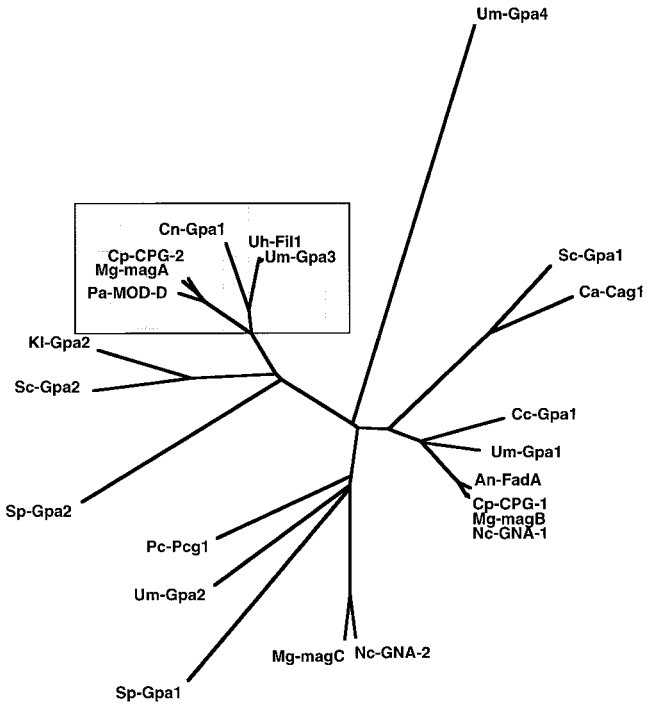

Figure 2.—Comparison of fungal G protein α subunits. The initial sequence alignment was produced by CLUSTAL W (Thompson *et al.* 1994) and the tree was then constructed using PROTDIST, FITCH, and DRAWTREE programs from the Phylogeny Inference package version 5.3c. (Felsenstein 1993). An-FadA is FadA from *A. nidulans* (Yu *et al.* 1996), Ca-Cag1 is Cag1 from *Candida albicans* (Sadhu *et al.* 1992), Cc-Gpa1 is Gpa1 from *Coprinus congregatus* (Kozak *et al.* 1995), Cn-Gpa1 is Gpa1 from *C. neoformans* (Tolkacheva *et al.* 1994), Cp-CPG-1 and Cp-CPG-2 are CPG-1 and CPG-2 from *C. parasitica* (Choi *et al.* 1995), Kl-Gpa2 is Gpa2 from *K. lactis* (Savinon-Tejeda *et al.* 1996), Mg-magA, Mg-magB, and Mg-magC are magA, magB, and magC from *M. grisea* (Liu and Dean 1997), Nc-GNA-1 and Nc-GNA-2 are GNA-1 and GNA-2 from *N. crassa* (Turner and Borkovich 1993), Pa-MOD-D is MOD-D from *P. anserina*, Pc-PCG1 is PCG1 from *Pneumocistis carinii* (Smulian *et al.* 1996), Sc-Gpa1 and Sc-Gpa2 are Gpa1 and Gpa2 from *S. cerevisiae* (Nakafuku *et al.* 1987, 1988), Sp-Gpa2 and Sp-Gpa1 are Gpa1 and Gpa2 from *S. pombe* (Obara *et al.* 1991; Isshiki *et al.* 1992), Uh-Fil1 is Fil1 from *U. hordei* (Lichter and Mills 1997), Um-Gpa1, Um-Gpa2, Um-Gpa3, and Um-Gpa4 are Gpa1, Gpa2, Gpa3, and Gpa4 from *U. maydis* (Regenfelder *et al.* 1997).

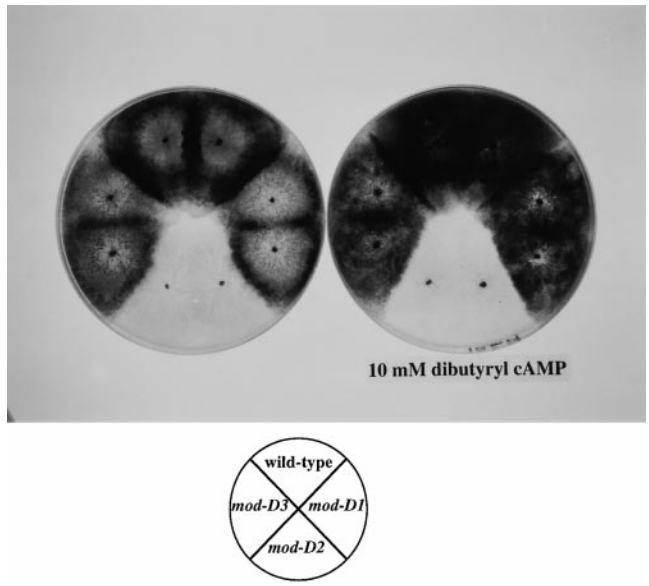

Figure 3.—Vegetative growth of the wild-type, *mod-D1*, *mod-D2*, and *mod-D3* strains on synthetic medium and synthetic medium supplemented with 10 mm dibutyryl cAMP.

mutated position. Nevertheless, whether the splicing occurs at these alternative positions or not, as in the large fragment, in all cases a stop codon is present immediately downstream from the end of the second exon. A truncated protein of 155 aa should be synthesized.

Previous studies on Gα subunits revealed that the sequences interacting with effectors are all located in the C-terminal half of the protein (for review see Conklin and Bourne 1993). This part of the protein should be absent in the MOD-D1 polypeptide, suggesting that the mutant protein would be unable to activate its effector.

The *mod-D1* and *mod-D3* mutant phenotypes can be partially restored by an increase of the level of cAMP: A possible relation between the function of *mod-D* and cyclic AMP was suggested by the cloning of the *PaAC* gene as a partial suppressor of the *mod-D1* mutant (Loubradou *et al.* 1996). To verify this relationship, the wild-type and *mod-D* mutant strains were grown under conditions that increase the cAMP level. The growth of the wild-type strain and of the three *mod-D* mutant strains was examined on synthetic medium supplemented with 10 mm dibutyryl cAMP, an analog of cAMP (Figure 3). On this medium, *mod-D1* and *mod-D3* mutant strains, filament network, and aerial filament density were clearly increased but no effect was observed on the phenotype of the *mod-D2* mutant. We noted an unexpected slight effect of the dibutyryl cAMP on the wild-type strain. In this case, unlike mutant strains, density of filament network and aerial filaments situated in the center of the colony was decreased. Nevertheless, on synthetic medium supplemented with 10 mm dibutyryl cAMP, the wild-type strain is less damaged than mutant strains. The dibutyryl cAMP effect does not seem to be an indirect effect unrelated to *mod-D* mutation since it has no effect on the *mod-D2* mutant strain. To confirm this, an inhibitor of phosphodiesterase, theophylline, was used to decrease cAMP hydrolysis. In *C. parasitica*, a close relative of *P. anserina*, this drug can induce an eightfold increase of the cAMP level, and this is a more efficient phosphodiesterase inhibitor than other xanthine derivatives such as caffeine or IBMX (3-isobutyl-1-methylxanthine; Chen *et al.* 1996). As for dibutyryl cAMP, partial restoration of a wild-type phenotype was obtained for *mod-D1* and *mod-D3* mutants when synthetic medium was supplemented with 4 mm theophylline

(data not shown). Once again, no restoration of *mod-D2* mutant growth was obtained in this medium.

These findings suggest that *mod-D1* and *mod-D3* mutations likely result in a decrease of cAMP concentration since an increase of the cAMP level partly restores the vegetative growth of these mutants. These results also support the idea that the suppressor effect on the *mod-D1* mutant of an additional ectopic copy of *PaAC* is due to its adenylate cyclase activity and not to any side effect.

MOD-D2 mutant protein is not constitutively active: The difference observed in response to cAMP suggests that the *mod-D1* and *mod-D2* mutations do not lead to the same functional defect. MOD-D1 would be defective in activating its effector. Is MOD-D2 constitutively activated? To answer the question, a mutation known to activate constitutively Gα proteins (Landis *et al.* 1989; Lyons *et al.* 1990) was generated using site-directed mutagenesis. The arginine residue at position 180 was replaced by a cysteine (Figure 1). The mutant allele was named *mod-D*^{R180C}. Protoplasts from wild-type strain were transformed with *mod-D*^{R180C} and the phenotype of the transformants was analyzed on synthetic medium. Strains containing this *mod-D* mutant allele display a very dense mycelial network with aerial filaments and exhibit an intense and premature pigmentation. This phenotype is the opposite of the mod-D2 phenotype, which is characterized by a defect in secondary and aerial hyphae formation and a lack of pigmentation. Therefore, it is unlikely that *mod-D2* encodes a constitutively activated protein. The phenotype due to *mod-D*^{R180C} also confirms the involvement of *mod-D* in the production of these differentiated mycelial structures since it is possible to obtain *mod-D* mutations that either strongly enhance or drastically reduce their formation.

The suppressor effect of *mod-D1* on vegetative incompatibility is not due to a decrease in the level of cAMP: The *mod-D1* mutation and the *mod-B* mutations were isolated as suppressors of the autolytic phenotype of the *het-C het-E mod-A1 mod-C1* strain (Labarère and Bernet 1979a). The *mod-D2* and *mod-D3* mutations were selected to inhibit the autolysis due to the *mod-B11* mutation (Durrens *et al.* 1979). These functional relations between *mod-D* and *mod-B* led us to test if the *mod-D* mutations, like the *mod-B* mutations, would suppress the cryosensitivity of the *het-C het-E mod-A1* strain (Bernet *et al.* 1973). The radial growth of the *het-C het-E mod-A1*, *het-C het-E mod-A1 mod-D1*, and *het-C het-E mod-A1 mod-D2* strains was compared at 11° (Figure 4). A restoration of growth is observed at this temperature for both *mod-D* mutations. However, this effect is less pronounced than the one noticed in the presence of the *mod-B1* mutation. The *het-C het-E mod-A1 mod-B1* strain exhibits after 25 days a radial growth that is ~80% of that of wild type at 11° (Boucherie 1979), but the rate is only 30% of wild-type growth for a *het-C het-E mod-A1 mod-D1* strain.

This effect of the *mod-D* mutations on vegetative incompatibility led us to investigate a possible involvement

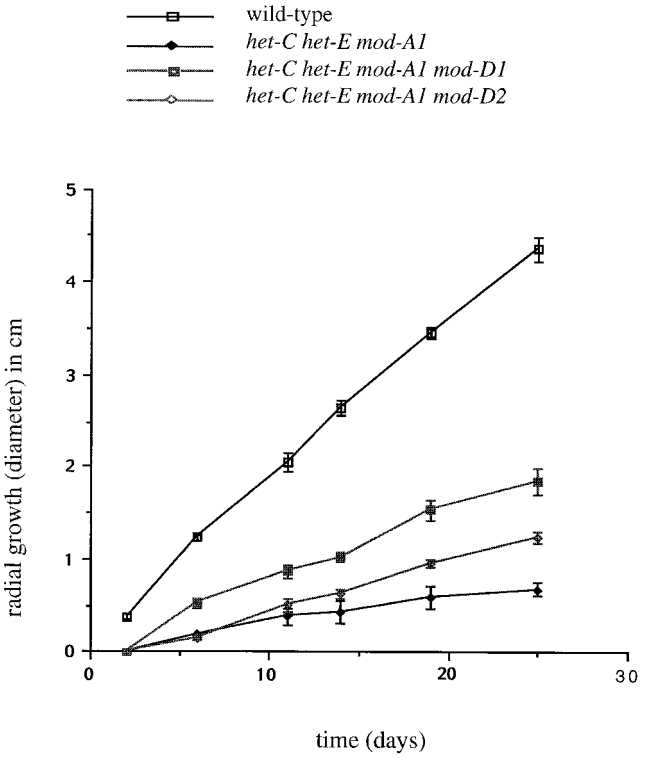

Figure 4.—Partial suppression of the cold sensitivity of the *het-C het-E mod-A1* strain by *mod-D1* and *mod-D2* mutations. The radial growth at 11° on D0 medium of the WT, *het-C het-E mod-A1*, *het-C het-E mod-A1 mod-D1*, and *het-C het-E mod-A1 mod-D2* strains was measured on a time basis. Each point corresponds to the average of four independent measures. Bars mark standard errors.

of cAMP on vegetative incompatibility. The wild-type, *het-C het-E mod-A1*, and *het-C het-E mod-A1 mod-D1* strains were grown at 11° on D0 medium supplemented or not with 10 mm dibutyryl cAMP. For all of the strains, the addition of dibutyryl cAMP stimulated growth independently of the presence of the *mod-D1* mutation (data not shown). The growth restoration of the *het-C het-E mod-A1 mod-D1* strain does not appear to be the consequence of a decreased level of cAMP. Therefore, this restoration is probably not linked to the defect in the cAMP pathway resulting from the *mod-D1* mutation.

DISCUSSION

The characterization of previously described *mod* genes in the fungus *P. anserina* has been undertaken to elucidate the molecular mechanism of vegetative incompatibility and to understand the relationship between this process and some developmental steps in this species. Three *mod-D* mutants have been isolated using two distinct screening procedures. These two procedures were designed for selecting mutations in genes that would be involved in the control of the cell lysis reaction due to vegetative incompatibility (Durrens *et al.* 1979; Labarère and Bernet 1979a). In this article, we report

the cloning of the *mod-D* gene. The MOD-D protein is a Gα subunit of a heterotrimeric G protein. Two other genes, which display functional interactions with *mod-D*, have been previously characterized: *mod-E* and *PaAC*. They encode, respectively, an HSP90 (Loubradou *et al.* 1997) and an adenylate cyclase (Loubradou *et al.* 1996), indicating that proteins encoded by *mod* genes belong to signal transduction pathways.

The mod-D2 phenotype may correspond to the loss of function of both MOD-D and Gβγ-associated subunits: All of the *mod-D1* cDNAs analyzed encode a polypeptide lacking the C-terminal half of the wild-type protein. This part of the protein is known to be involved in the binding to the effectors (for review see Conklin and Bourne 1993), suggesting that the MOD-D1 protein would have lost this property. The *mod-D2* mutant displays more altered defects in development than a *mod-D1* strain. Unlike *mod-D1*, none of the defects displayed by the *mod-D2* mutant strain can be restored by an increase of the cAMP level. Furthermore, a constitutively active MOD-D mutant protein induces a phenotype that is opposite to the *mod-D2* mutant phenotype; thus, the *mod-D2* mutant does not correspond to a constitutive activation of the G protein. The differences observed as the consequence of *mod-D1* and *mod-D2* mutations could then be explained by a different behavior of the Gα mutant proteins toward the βγ subunits. As the C-terminal end of Gα proteins is important for the binding to the receptor (for review see Conklin and Bourne 1993), a hypothesis could be that the MOD-D2 protein is unable to bind to the receptor. The additional amino acids present in the C-terminal part of MOD-D2, due to the suppression of the stop codon by the mutation, could induce some structural modifications preventing the binding. In this hypothesis, the mod-D2 phenotype would correspond to both loss of MOD-D activation and loss of Gβγ subunits activation. This hypothesis would also imply that both α and βγ subunits have effectors. Such a result has already been reported in *Aspergillus nidulans*. A strain, in which the *fadA* mutant gene encoding a Gα subunit inhibits the release of Gβγ subunits, exhibits a more-altered phenotype than the strain in which the *fadA* gene has been deleted (Yu *et al.* 1996).

***mod-D* and vegetative incompatibility: evidence for a second pathway controlled by *mod-D*:** The effect of *mod-D* mutations on vegetative incompatibility was investigated on the cryosensitive *het-C het-E mod-A1* incompatible strain. This strain is unable to grow at 11°. The *mod-A1* mutant strain and the wild-type strain are able to grow at 11°. The growth inhibition is due to the presence of the two *het-C* and *het-E* incompatible genes. In the presence of either *mod-D1* or *mod-D2* mutations, the growth of the cryosensitive *het-C het-E mod-A1* strain is partly restored at 11°. This result confirms the involvement of the *mod-D* gene in vegetative incompatibility. The *het-C*, *het-E*, and *mod-A* genes have been isolated and sequenced. They encode, respectively, a protein with identity to a glycolipid transfer protein (Saupe *et al.* 1994), a protein with a functional GTP-binding site and WD40 repeats (Saupe *et al.* 1995; Espagne *et al.* 1997), and a protein that contains potential SH3-binding sites (Barreau *et al.* 1998). The functional interactions between these genes remain unclear but their sequences reveal a probable involvement in signal transduction in agreement with our results.

An increase of the cAMP level does not attenuate the growth restoration due to the *mod-D1* mutation in the *het-C het-E mod-A1 mod-D1* strain. This result seems to indicate that the growth restoration observed is not due to a decrease in the cAMP level resulting from the presence of the *mod-D1* mutation. It also indicates that the *mod-D1* mutation induces some defects in a signaling pathway distinct from the cAMP pathway. The characterization of this second pathway responsible for the *mod-D1* effect on vegetative incompatibility is now under investigation.

A family of fungal Gα subunits involved in cAMP signal transduction pathway: MOD-D from *P. anserina*, magA from *M. grisea*, CPG-2 from *C. parasitica*, Gpa3 from *U. maydis*, Fil1 from *U. hordei*, and Gpa1 from *C. neoformans* may define a new family of Gα proteins. They are characterized by a highly conserved C-terminal region. The C-terminal region of Gα proteins is involved in effector and receptor binding (for review see Conklin and Bourne 1993). This family of Gα proteins may interact with the same type of receptor and effector. The hypothesis of a conserved pathway for these Gα proteins is not based only on sequence similarity but also on the positive regulatory role of MOD-D, CPG-2, Gpa3, Fil1, and Gpa1 on the level of cAMP. The disorders in *mod-D1* and *mod-D3* strains during vegetative growth can be restored by an increase of the cAMP level. In the same way, the strains deleted for *gpa1*, *gpa3*, *Fil1*, and *cpg-2* are deficient in cAMP production (Choi *et al.* 1995; Gao and Nuss 1996; Mills *et al.* 1996; Alspaugh *et al.* 1997; Kahmann and Basse 1997). It is also interesting to note that the most closely related proteins to this family are proteins encoded by *GPA2* genes from the yeasts *S. cerevisiae*, *S. pombe*, and *K. lactis*; the function of these proteins is to regulate positively the cAMP level (Isshiki *et al.* 1992; Savinon-Tejeda *et al.* 1996; Kübler *et al.* 1997; Lorenz and Heitman 1997).

The MOD-D protein is involved in both the sexual cycle of *P. anserina* (by controlling key steps in protoperithecia formation and spore germination) and vegetative development (Durrens *et al.* 1979; Labarère and Bernet 1979a,b). The involvement of Gpa3 from *U. maydis* and GPA1 from *C. neoformans* in pathogenicity and in the mating reaction has been demonstrated (Alspaugh *et al.* 1997; Regenfelder *et al.* 1997). This mating function is linked to the cAMP pathway in *C. neoformans* (Alspaugh *et al.* 1997). The phenotype described for the strain deleted for *Fil1* is in accordance with such functions, and this gene also interferes with the dimor-

phic switch in *U. hordei* (Lichter and Mills 1997). No clear information is available so far on the physiological function of *cpg-2*. Surprisingly, it seems to be dispensable for virulence and both sexual and asexual reproduction. These steps are controlled by *cpg-1*, the other Gα subunit characterized in *C. parasitica* (Choi *et al.* 1995; Chen *et al.* 1996; Gao and Nuss 1996). A similar situation is observed in *M. grisea*. The magA protein appears to be necessary only for ascospore maturation and/or germination, while the magB polypeptide that shares 99% identity with CPG-1 seems to control many aspects of the life cycle, including a well-defined cAMP-dependent step involving appressorium formation (Liu and Dean 1997). CPG-1 negatively regulates the adenylate cyclase, as expected from a protein that belongs to the fungal Gαi family (Gao and Nuss 1996), but unexpectedly, *mag*B deletion seems to induce a decrease of the cAMP level. A strain in which *mag*B has been inactivated can recover appressorium formation if external cAMP or phospodiesterase inhibitor is added (Liu and Dean 1997). This result is in contradiction with the results of the phylogenetic analysis but it may be due to an indirect effect of the mutation. For example, the cells containing the *mag*B mutation may need a higher level of cAMP than the wild type to undergo appressorium formation.

From all of these data, it is difficult to propose a model for the function of the signal transduction cascade controlled by MOD-D family Gα subunit proteins. This may be due to the natural environment diversity of these species. The signals dependent on these Gα proteins may be very different for a fungus living in animal brains, on plant leaves, or on herbivore dungs.

The growing interest in fungi signal transduction and the key role of Gα proteins in this family will certainly lead to a rapid increase of family members. The study of these genes in our laboratory and others will allow determination of the precise characteristics of this potentially conserved pathway. In this respect, *P. anserina* is an excellent paradigm since there is evidence for the existence of a second signaling transduction pathway regulated by *mod-D* and a possible active role for Gβγ subunits.

This work was supported by a grant from the Association pour la Recherche sur le Cancer. G.L. was supported by a fellowship from the Association pour la Recherche sur le Cancer.

LITERATURE CITED

Akins, R. A., and A. M. Lambowitz, 1985 General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. **5:** 2272–2278.

Alspaugh, J. A., J. R. Perfect and J. Heitman, 1997 *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP. Genes Dev. **11:** 3206–3217.

Arganoza, M. T., J. Ohrnberger, J. Min and R. A. Akins, 1994 Suppressor mutants of *Neurospora crassa* that tolerate allelic differences at single or at multiple heterokaryon incompatibility loci. Genetics **137:** 731–742.

Ballance, D. J., 1991 Transformation systems for filamentous fungi and an overview of fungal gene structure, pp. 1–29, in *Molecular Industrial Mycology*, edited by S. A. Leong and R. M. Berka. Marcel Dekker Inc., New York.

Barreau, C., M. Iskandar, G. Loubradou, V. Levallois and J. Bégueret, 1998 The *mod-A* suppressor of non-allelic heterokaryon incompatibility in *Podospora anserina* encodes a proline-rich polypeptide involved in female organs formation. Genetics **149:** 915–926.

Bégueret, J., B. Turcq and C. Clavé, 1994 Vegetative incompatibility in filamentous fungi: *het* genes begin to talk. Trends Genet. **10:** 441–446.

Belcour, L., and J. Bernet, 1969 Sur la mise en évidence d'un gène dont la mutation supprime spécifiquement certaines manifestations d'incompatibilité chez le *Podospora anserina*. C. R. Acad. Sci. Paris **269:** 712–714.

Bergès, T., and C. Barreau, 1989 Heat-shock at an elevated temperature improves transformation efficiency of protoplasts from *Podospora anserina*. J. Gen. Microbiol. **135:** 601–604.

Bernet, J., 1971 Sur un cas de suppression de l'incompatibilité cellulaire chez le champignon *Podospora anserina*. C. R. Acad. Sci. Paris **273:** 1120–1122.

Bernet, J., J. Bégueret and J. Labarère, 1973 Incompatibility in the fungus *Podospora anserina*. Are the mutations abolishing the incompatibility reaction ribosomal mutation? Mol. Gen. Genet. **124:** 35–50.

Boucherie, H., 1979 L'incompatibilité protoplasmique chez *Podospora anserina*: caractérisation et regulation des variations physiologiques associées, relation avec la différenciation des organes reproducteurs femelles. Ph.D. Thesis, University of Bordeaux II, Bordeaux, France.

Boucherie, H., J. Bégueret and J. Bernet, 1976 The molecular mechanism of protoplasmic incompatibility and its relationship to the formation of protoperithecia in *Podospora anserina*. J. Gen. Microbiol. **92:** 59–66.

Bourne, H. R., D. A. Sanders and F. McCormick, 1991 The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:** 117–127.

Bruchez, J. J. P., J. Eberle and V. E. A. Russo, 1993 Regulatory sequences in the transcription of Neurospora crassa genes: CAAT box, TATA box, introns, poly(A) tail formation sequences. Fungal Genet. Newsl. **40:** 89–96.

Chen, B., S. Gao, G. H. Choi and D. L. Nuss, 1996 Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. Proc. Natl. Acad. Sci. USA **93:** 7996–8000.

Choi, G. H., B. Chen and D. L. Nuss, 1995 Virus-mediated or transgenic suppression of a G-protein α subunit and attenuation of fungal virulence. Proc. Natl. Acad. Sci. USA **92:** 305–309.

Conklin, B. R., and H. R. Bourne, 1993 Structural elements of Gα subunits that interact with Gβγ, receptors, and effectors. Cell **73:** 631–641.

Csermely, P., T. Schnaider, C. Soti, Z. Prohaszka and G. Nardai, 1998 The 90-kDA molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol. Ther. **79:** 129–168.

Durrens, P., 1982 *Podospora anserina* mutants defective in cell regeneration and ascospore germination: the presence of a possible lesion of the plasma membrane. Exp. Mycol. **6:** 216–224.

Durrens, P., 1984 *Podospora anserina* mutation reducing cell survival under glucose starvation. Exp. Mycol. **8:** 342–348.

Durrens, P., and J. Bernet, 1982 *Podospora anserina* mutations inhibiting several development alternatives and growth renewal. Curr. Genet. **5:** 181–186.

Durrens, P., F. Laigret, J. Labarère and J. Bernet, 1979 *Podospora anserina* mutant defective in protoperithecium formation, ascospore germination, and cell regeneration. J. Bacteriol. **140:** 835–842.

Espagne, E., P. Balhadère, J. Bégueret and B. Turcq, 1997 Reactivity in vegetative incompatibility of the HET-E protein of the fungus *Podospora anserina* is dependent on GTP-binding activity and WD40 repeated domain. Mol. Gen. Genet. **256:** 620–627.

Esser, K., 1974 *Podospora anserina*, pp. 531–551 in *Handbook of Genetics*, edited by R. C. King. Plenum Press, New York.

Felsenstein, J., 1993 PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.

Gao, S., and D. L. Nuss, 1996 Distinct roles for two G protein α subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. Proc. Natl. Acad. Sci. USA **93:** 14122–14127.

Glass, N. L., and G. A. Kuldau, 1992 Mating type and vegetative incompatibility in filamentous ascomycetes. Annu. Rev. Phytopathol. **30:** 201–224.

Glass, N. L., S. J. Vollmer, C. Staben, J. Grotelueschen, R. L. Metzenberg *et al.*, 1988 DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. Science **241:** 570–573.

Glass, N. L., J. Grotelueschen and R. L. Metzenberg, 1990 *Neurospora crassa A* mating type region. Proc. Natl. Acad. Sci. USA **87:** 4912–4916.

Isshiki, T., N. Mochizuki, T. Maeda and M. Yamamoto, 1992 Characterization of a fission yeast gene *gpa2*, that encodes a Gα subunit involved in the monitoring of nutrition. Genes Dev. **6:** 2455–2462.

Kahmann, R., and C. Basse, 1997 Signaling and development in pathogenic fungi—new strategies for plant protection? Trends Plant Sci. **2:** 366–368.

Kozak, K. R., L. M. Foster and I. K. Ross, 1995 Cloning and characterization of a G protein alpha-subunit-encoding gene from the basidiomycete, *Coprinus congregatus*. Gene **163:** 133–137.

Kübler, E., H. U. Mosch, S. Rupp and M. P. Lisanti, 1997 Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J. Biol. Chem. **272:** 20321–20323.

Labarère, J., and J. Bernet, 1977 Protoplasmic incompatibility and cell lysis in *Podospora anserina*. I. Genetic investigation on mutations of a novel modifier gene that suppresses cell destruction. Genetics **87:** 249–257.

Labarère, J., and J. Bernet, 1979a A pleiotropic mutation affecting protoperithecium formation and ascospore outgrowth in *Podospora anserina*. J. Gen. Microbiol. **113:** 19–27.

Labarère, J., and J. Bernet, 1979b Protoplasmic incompatibility in *Podospora anserina*: a possible role for its associated proteolytic activity. Genetics **93:** 525–537.

Landis, C. A., S. B. Masters, A. Spada, A. M. Pace, H. R. Bourne *et al.*, 1989 GTPase inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumors. Nature **340:** 692–696.

Lecellier, G., and P. Silar, 1994 Rapid methods for nucleic acids extraction from Petri dish-grown mycelia. Curr. Genet. **25:** 122–123.

Lichter, A., and D. Mills, 1997 Fil1, a G-protein α-subunit that acts upstream of cAMP and is essential for dimorphic switching in haploid cells of *Ustilago hordei*. Mol. Gen. Genet. **256:** 426–435.

Liu, S., and R. A. Dean, 1997 G protein alpha subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. Mol. Plant-Microbe Interact. **10:** 1075–1086.

Lorenz, M. C., and J. Heitman, 1997 Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. EMBO J. **16:** 7008–7018.

Loubradou, G., J. Bégueret and B. Turcq, 1996 An additional copy of the adenylate cyclase gene relieves developmental defects produced by a mutation in a gene involved in vegetative incompatibility in *Podospora anserina*. Gene **170:** 119–123.

Loubradou, G., J. Bégueret and B. Turcq, 1997 A mutation in an *HSP90* gene affects the sexual cycle and suppresses vegetative incompatibility in the fungus *Podospora anserina*. Genetics **147:** 581–588.

Lyons, J., C. A. Landis, G. Harsh, L. Vallar, K. Grünewald *et al.*, 1990 Two G protein oncogenes in human endocrine tumors. Science **249:** 655–659.

Mills, D., J. Agnan and K. McCluster, 1996 Cyclic AMP controls dimorphic switching of *Ustilago hordei*, pp. 83–92 in *Molecular Aspect of Pathogenicity and Resistance: Requirement for Signal Transjyduction*, edited by D. Mills, H. Kunoh, N. T. Keenand and S. Mayama. APS Press, St. Paul.

Nakafuku, M., H. Itoh, S. Nakamura and Y. Kaziro, 1987 Occurrence in *Saccharomyces cerevisiae* of a gene homologous to the cDNA coding for the alpha-subunit of mammalian G proteins. Proc. Natl. Acad. Sci. USA **84:** 2140–2144.

Nakafuku, M., T. Obara, K. Kaibuchi, I. Miyajima, A. Miyajima *et al.*, 1988 Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc. Natl. Acad. Sci. USA **85:** 1374–1378.

Neer, E. J., 1995 Heterotrimeric G proteins: organizers of transmembrane signals. Cell **80:** 249–257.

Newmeyer, D., 1970 A suppressor of the heterokaryon-incompatibility associated with mating type in *Neurospora crassa*. Can. J. Genet. Cytol. **12:** 914–926.

Obara, T., M. Nakafuku, M. Yamamoto and Y. Kaziro, 1991 Isolation and characterization of a gene encoding a G-protein alpha subunit from *Schizosaccharomyces pombe*: involvement in mating and sporulation pathways. Proc. Natl. Acad. Sci. USA **88:** 5877–5881.

Orbach, M. J., A. Sweigard, A. Walter, L. Farral, F. G. Chumley *et al.*, 1991 Strategies for the isolation of the avirulence genes from the rice blast *Magnaporthe grisea*. Fungal Genet. Newsl. **38:** 16.

Ponce, M. R., and J. L. Micol, 1992 PCR amplification of long DNA fragments. Nucleic Acids Res. **20:** 663.

Regenfelder, E., T. Spellig, A. Hartmann, S. Lauenstein, M. Bölker *et al.*, 1997 G proteins in *Ustilago maydis*: transmission of multiple signals? EMBO J. **16:** 1934–1942.

Rizet, G., and C. Engelman, 1949 Contribution à l'étude d'un ascomycète tétrasporé: *Podospora anserina*. Rev. Cytol. et Biol. Veg. **11:** 201–304.

Sadhu, C., D. Hoekstra, M. J. McEachern, S. I. Reed and J. B. Hicks, 1992 A G-protein alpha subunit from asexual *Candida albicans* functions in the mating signal transduction pathway of *Saccharomyces cerevisiae* and is regulated by the a1-alpha-2 repressor. Mol. Cell. Biol. **12:** 1977–1985.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higushi *et al.*, 1988 Primed-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:** 487–491.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen and A. R. Coulson, 1977 DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

Saupe, S., C. Descamps, B. Turcq and J. Bégueret, 1994 Inactivation of the *Podospora anserina* vegetative incompatibility locus *het-c*, whose product resembles a glycolipid transfer protein, drastically impairs ascospore production. Proc. Natl. Acad. Sci. USA **91:** 5927–5931.

Saupe, S., B. Turcq and J. Bégueret, 1995 A gene responsible for vegetative incompatibility in the fungus *Podospora anserina* encodes a protein with a GTP-binding motif and Gβ homologous domain. Gene **162:** 135–139.

Saupe, S. J., G. A. Kuldau, M. L. Smith and N. L. Glass, 1996 The product of the *het-C* heterokaryon incompatibility gene of *Neurospora crassa* has characteristics of a glycine-rich cell wall protein. Genetics **143:** 1589–1600.

Savinon-Tejeda, A. L., L. Ongay-Larios, J. Ramirez and R. Coria, 1996 Isolation of a gene encoding a G protein alpha subunit involved in the regulation of cAMP levels in the yeast *Kluyveromyces lactis*. Yeast **12:** 1125–1133.

Simon, M. I., M. P. Strathmann and N. Gautam, 1991 Diversity of G proteins in signal transduction. Science **252:** 802–808.

Smulian, A. G., M. Ryan, C. Staben and M. Cushion, 1996 Signal transduction in *Pneumocystis carinii*: characterization of the genes (*pcg1*) encoding the alpha subunit of the G protein (PCG1) of *Pneumocystis carinii carinii* and *Pneumocystis carinii ratti*. Infect. Immun. **64:** 691–701.

Staben, C., and C. Yanofsky, 1990 *Neurospora crassa a* mating type region. Proc. Natl. Acad. Sci. USA **87:** 4917–4921.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Tolkacheva, T., P. McNamara, E. Piekarz and W. Courchesne, 1994 Cloning of a *Cryptococcus neoformans* gene, *GPA1*, encoding a G-protein α-subunit homolog. Infect. Immun. **62:** 2849–2856.

Turcq, B., and J. Bégueret, 1987 The *ura5* gene of the filamentous fungus *Podospora anserina*: nucleotide sequence and expression in transformed strains. Gene **53:** 201–209.

Turcq, B., M. Denayrolles and J. Bégueret, 1990 Isolation of the

two allelic incompatibility genes *s* and *S* of the fungus *Podospora anserina*. Curr. Genet. **17:** 297–303.

Turner, G. E., and K. A. Borkovich, 1993 Identification of a G protein α subunit from *Neurospora crassa* that is a member of the G_i family. J. Biol. Chem. **268:** 14805–14811.

Vellani, T. S., A. J. Griffiths and N. L. Glass, 1994 New mutations that suppress mating-type vegetative incompatibility in *Neurospora crassa*. Genome **37:** 249–255.

Yu, J.-H., J. Weiser and T. H. Adams, 1996 The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J. **15:** 5184–5190.

Communicating editor: J. J. Loros