Evidence for a Role of the Regulator of G-Protein Signaling Protein CPRGS-1 in G_α_ Subunit CPG-1-Mediated Regulation of Fungal Virulence, Conidiation, and Hydrophobin Synthesis in the Chestnut Blight Fungus _Cryphonectria parasitica_

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We previously reported that the chestnut blight fungus _Cryphonectria parasitica_ expresses at least three G-protein α subunits and that G_α_ subunit CPG-1 is essential for regulated growth, pigmentation, sporulation, and virulence. We now report the cloning and characterization of a _C. parasitica_ regulator of G-protein signaling (RGS) protein, CPRGS-1. The phylogenetic relationship of CPRGS-1 to orthologs from other fungi was inferred and found to be generally concordant with species relationships based on 18S ribosomal sequences and on morphology. However, Hemiascomycotina RGS branch lengths in particular were longer than for their 18S sequence counterparts, which correlates with functional diversification in the signaling pathway. Deletion of _cprgs-1_ resulted in reduced growth, sparse aerial mycelium, and loss of pigmentation, sporulation, and virulence. Disruption of _cprgs-1_ was also accompanied by a severe posttranscriptional reduction in accumulation of CPG-1 and G_β_ subunit CPGB-1 and severely reduced expression of the hydrophobin-encoding gene cryparin. The changes in phenotype, cryparin expression, and CPGB-1 accumulation resulting from _cprgs-1_ gene deletion were also observed in a strain containing a mutationaly activated copy of CPG-1 but not in strains containing constitutively activated mutant alleles of the other two identified G_α_ subunits, CPG-2 and CPG-3. Furthermore, _cprgs-1_ transcript levels were increased in the activated CPG-1 strain but were unaltered in activated CPG-2 and CPG-3 strains. The results strongly suggest that CPRGS-1 is involved in regulation of G_α_ subunit CPG-1-mediated signaling and establish a role for a RGS protein in the modulation of virulence, conidiation, and hydrophobin synthesis in a plant pathogenic fungus.

Heterotrimeric G proteins play an essential role in the ability of eukaryotic cells to respond to extracellular signals. Activation of the pathway occurs when extracellular ligands bind to transmembrane G-protein-coupled receptors. This allows the G_α_ subunit of the heterotrimeric G-protein complex to exchange GDP for GTP, which results in dissociation of the G_α_ and G_βγ_ subunits and activation of downstream effectors by both G_α_ and G_βγ_ (for a review, see reference 22). G_α_ and G_β_ subunits have been identified in numerous filamentous fungi, and their importance in biological processes such as morphogenesis, asexual and sexual reproduction and pathogenesis has been firmly established (reviewed in 2 and 34).

An essential step in controlling the cellular response to G-protein signal transduction is termination of the signal, which is achieved through the intrinsic GTPase activity of the G_α_ subunit. The GTPase activity is relatively weak (21), and is greatly enhanced by interaction of the G_α_ subunit with regulator of G-protein signaling (RGS) proteins (reviewed in reference 47). Evidence for the existence of RGS proteins came initially from studies of the mating pheromone response in _Saccharomyces cerevisiae_, which is mediated via heterotrimeric G proteins. The RGS-encoding SST2 gene was shown to be indispensable for desensitization of the signaling pathway, allowing cells that fail to mate to resume normal growth and cell division (reviewed in reference 32). Sst2 was isolated in a complex with Gpa1 by immunoprecipitation, providing evidence for a direct interaction between Sst2 and Gpa1 (14). Subsequent studies in a range of eukaryotic organisms have led to the recognition of a gene family that shares an ~130-amino-acid domain, termed the RGS domain. Many RGS proteins contain N- or C-terminal extensions with different functions, linking them to other signaling pathways. Several reviews have been published that focus on RGS proteins and their diverse roles in cellular functions (12, 26, 47).

Studies on the role of RGS proteins in regulation of G-protein signaling in filamentous fungi is currently limited to the model ascomycete _Aspergillus nidulans_ (23, 33, 58) and the homobasidiomycete _Schizophyllum commune_ (18). The FlbA RGS protein of _A. nidulans_, the second RGS protein to be identified as such (33), has been shown to negatively regulate signaling for vegetative growth and activate asexual sporulation, mediated through the G_α_ subunit FadA and the G_β_ subunit SfaD (46, 58). Han et al. (23) recently described a second RGS protein in _A. nidulans_, RgsA, that appears to dampen stress responses and stimulate asexual sporulation through negative regulation of the G_α_ subunit Gnb. The RGS protein encoded by the _thr-1_ gene of _S. commune_ has been shown to be required for aerial-hyphae formation in monokaryons and fruiting body formation in dikaryons (18). Recent characterizations of constitutively activated G_α_ subunits in _S. commune_ suggest the possibility that Thr-1 may regulate the activity of...
two Ga subunits, ScGP-A and ScGP-C (55). To further our understanding of the role of RGS proteins in fungal pathogenesis, we now report the cloning and characterization of the RGS-encoding gene, cprgs-1 from the chestnut blight pathogen Cryphonectria parasitica.

Previous studies on G-protein signaling in *C. parasitica* identified genes encoding three Ga subunits (cpg-1, cpg-2, and cpg-3), one Gb subunit (cpgb-1), and a regulator of Gβ function (bdm-1) (19, 28, 29, 40). Germline deletion experiments revealed that Ga subunit CPG-1 is essential for growth, conidiation, pigmentation, and virulence, whereas Gβ-2 is dispensable (19). A recently isolated third Ga subunit, CPG-3, awaits detailed characterization (40). The Gβ subunit CPGB-1 was found to be dispensable for growth, since Δcpgb-1 strains grew even faster than a wild-type strain. However, pigmentation, sporulation, and virulence were severely reduced, indicating a role for CPGB-1 in these processes (28). In a recent study, CPG-1 was further characterized by introducing mutations designed to abolish GTPase activity, resulting in constitutive activation. The resulting phenotypic changes resembled those caused by a cpg-1 deletion. Both mutant strains showed reduced growth and pigmentation, failed to produce conidia, and were completely avirulent, indicating that tight regulation of CPG-1-mediated signaling is required to control these processes. In contrast, opposing responses of Δcpg-1 and activated mutant strains were found to chronic heat, hyperosmolarity, and oxidative stress. Also, transcript levels of the hydrophobin-encoding gene cryparin were increased in the Δcpg-1 strain but nearly undetectable in mutants expressing activated CPG-1, suggesting a direct role for CPG-1 in regulating cryparin gene expression (49).

The characterization of cprgs-1 described here strongly suggests that CPG-1 is a target of CPRGS-1 regulation and establishes a role for an RGS protein in the modulation of virulence, conidiation, and hydrophobin synthesis in a plant pathogenic fungus. A posttranscriptional reduction in the accumulation of CPG-1 and the Gβ subunit CPGB-1 was also observed in the cprgs-1 deletion mutant, reinforcing our previous reports that mutations in fungal G-protein signaling components can have a dramatic effect on the accumulation of presumptive interacting components (29, 40, 49).

**MATERIALS AND METHODS**

Fungal strains, growth conditions, and transformation. *C. parasitica* strains were maintained on potato dextrose agar (PDA; Difco) as described previously (24). Preparative cultures for protein or RNA isolation were grown for 7 days at room temperature under ambient light, with cellophane covering the growth medium to facilitate mycelial harvest. Preparation and transformation of *C. parasitica* spheroplasts was carried out essentially as described by Churchill et al. (7). Benomyl (0.5 μg/ml) or hygromycin (40 μg/ml) were included in the growth medium to provide for selection of transformants. Pathogenicity assays were performed as described by Choi and Nuss (6), with 10 replicates per strain.

**Nucleic acid procedures.** To isolate the *C. parasitica* RGS homolog, a PCR was carried out by using a Platinum Taq DNA Polymerase High-Fidelity Supermix (Invitrogen, Carlsbad, Calif.) with degenerate primers RGS-F2 and RGS-R3 (see Table 1) and *C. parasitica* genomic DNA as a template. After a denaturing step of 3 min at 94°C, PCR amplification was carried out for 35 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C. The resulting 260-nucleotide PCR fragment was cloned into plasmid vector pUC19-Script Amp cloning kit (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions and sequenced. This fragment was used subsequently to screen the cDNA library described in Dawe et al. (10), which yielded several cprgs-1 encoding cDNA clones, none of which contained the complete open reading frame.

The Smart-RACE kit (Clontech/BD Biosciences, Bedford, Mass.) was used according to the manufacturer’s specifications to isolate the 5′ portion of the cprgs-1 cDNA. General molecular biology procedures and screening of genomic and cDNA libraries were carried out by standard techniques (48).

A cprgs-1 cDNA fragment was used to screen a *C. parasitica* genomic library, and a 6.2-kb KpnI/Xbal genomic DNA fragment was subcloned into pBluescript, resulting in plasmid pRGSX. The insert was fully sequenced and shown to contain the complete cprgs-1 open reading frame, as well as 2.7 kb upstream of the start codon and 1.5 kb downstream of the stop codon (GenBank accession number AF167360). To generate a gene replacement construct, a 1,030-bp SpII/SmaI fragment within the cprgs-1 open reading frame was replaced with a gene cassette conferring hygromycin resistance (9), generating plasmid pRGSX-Hyg. The insert was released from the vector by digestion with KpnI/NotI, and the digestion mixture was used to transform wild-type strain EP155. The Smart-RACE kit (Clontech/BD Biosciences, Bedford, Mass.) was used according to the manufacturer’s specifications to isolate the 5′ portion of the cprgs-1 cDNA. General molecular biology procedures and screening of genomic and cDNA libraries were carried out by standard techniques (48).

The expression vectors used in the present study were based on pCPXHY1 (Qbiogene, Carlsbad, Calif.) according to the manufacturer’s instructions. Quantitative analysis of transcript accumulation was performed via reverse transcription and quantitative PCR as described previously (39) by using TaqMan reagents (Applied Biosystems, Foster City, Calif.) and a GeneAmp 5700 PCR apparatus. Transcripts were analyzed at least twice, in triplicate on each occasion, from at least two independent RNA preparations, with primers and probes specific for 18S rRNA, cprgs-1, cpg-1, cpgb-1, and cryparin (39, 40) (Table 1). Calculations to obtain transcript accumulation values in the mutants relative to those in EP155 were performed by using the comparative ΔCt method as described previously (39) using the 18S rRNA values to normalize for variations in template concentration.

The expression vectors used in the present study were based on pCPXHY1 (8). The *C. parasitica* GPD promoter and terminator region from pCPXHY1 were cloned into pGem4Z (Promega, Madison, Wis.), and the original polyclinker between the GPD promoter and terminator was replaced with a polynucleotide containing HindIII, HpaI, NotI, and SpII restriction sites. To make pCPX-Hy3, the Hyg cassette from pCPXHY1 was cloned at the 5′ end of the GPD promoter. Plasmid pCPX-NBm2 was constructed by inserting the Neurospora crassa benomyl cassette (38) upstream of the GPD promoter.

**TABLE 1. Oligonucleotides used in this study**

| Purpose and oligonucleotide | Sequence (5′-3′) |
|----------------------------|-----------------|
| PCR-based cloning of cprgs-1 |                 |
| RGS-F2               | TGYGARYTNAAYATHGAYCA |
| RGS-R3               | TDAAYTNTNGNACGTYARTC |
| Mutated alleles of cpg-2 and cpg-3 |         |
| C2-QLF               | GTTGGAGAGTACGAAAGCGAG |
| C2-QLR               | CTCCGCTTCGAATCTCCACAC |
| C3-QLF               | GTCGGTTGGGCTCGATGCAAG |
| C3-QLR               | C1FSGATCGCAACCCACGGC |
| C2-FH                | TATAGCGTCAAGTGCTGCTGCG |
| C3-FH                | TATAGCGTACCCAATGCTGGC |
| C3-RS                | TATAGCGTACCATGCTGAGC |
| S-tag constructs     |                 |
| Stag-NF              | TATATGCAAAAAAGGACGGTCTGTC |
| Stag-NR              | ATACTCGAAGGTCGTCATGGCG |
| RGS-HF               | ATAAAGCTTCACTTCGAAATTCG |
| RGS-NR               | ATACGGGGGCATGCGTGAAAGGGC |
| Real-time RT-PCR     |                 |
| Cwyn-probe           | TCTACTGTGCGGCCAGCGCATCGCCG |
| Cwyn-F               | CAGCGCATACGAGCTGCG |
| Cwyn-R               | CGTGCAGACCTGTGCTGCTG |
| Cwyn-probe           | ACGCCAAACGGCACAGGTACCTGCA |
| Cwyn-F               | ACGCACAAGCAGCTTCCATTAA |
| Cwyn-R               | CGCGAGTTACGACCGATTG  |

* Nucleotides in boldface introduce the desired mutation. Underlined nucleotides indicate restriction sites used for cloning.

The expression vectors used in the present study were based on pCPXHY1 (8). The *C. parasitica* GPD promoter and terminator region from pCPXHY1 were cloned into pGem4Z (Promega, Madison, Wis.), and the original polyclinker between the GPD promoter and terminator was replaced with a polynucleotide containing HindIII, HpaI, NotI, and SpII restriction sites. To make pCPX-Hy3, the Hyg cassette from pCPXHY1 was cloned at the 5′ end of the GPD promoter. Plasmid pCPX-NBm2 was constructed by inserting the Neurospora crassa benomyl cassette (38) upstream of the GPD promoter.
To make a cprgs-1 overexpression construct, the cprgs-1 open reading frame was amplified by PCR with primers RGS-HF and RGS-NR (Table 1) using a full-length cDNA clone as a template. The PCR fragment was digested with HindIII and NotI and cloned into HindIII/NotI-digested pCPX-NBn2. The unique NsiI site at the start codon of the cprgs-1 open reading frame was used to introduce a S-tag (31), which was amplified by PCR from plasmid vector pET-31b (Novagen/EMD Biosciences, San Diego, Calif.) with the primers Stag-NF and Stag-NR (Table 1), resulting in plasmid pRSO. The S-tag was also introduced into the unique NsiI site in genomic clone pRGSX. The plasmids were sequenced to confirm the absence of mutations introduced by PCR.

PCR products were used as templates for introduction of cpg-2 and cpg-3 were performed as described previously (49) (with the PCR primers listed in Table 1 and cDNA clones of cpg-2 and cpg-3 as templates). The PCR products were digested with HindIII and SphI. The mutated cpg-2 PCR fragment was cloned into pCPX-NBn2, resulting in plasmid pCPX-C2QL, and the cpg-3 PCR fragment was cloned into pCPX-Hy3, resulting in plasmid pCPX-C3QL. The coding regions were completely sequenced to ensure that only the desired mutations had been introduced. Plasmid pPCX-C2QL was transformed into E. coli strain (19), and plasmid pCPX-C3QL was transformed into EP155.

DNA sequencing was performed by the CBR sequencing facility using the ABI Prism BigDye Terminator Ready-Reaction Cycle Sequencing kit (Applied Biosystems) to prepare the sequencing reactions, which were analyzed on an ABI 3100 genetic analyzer (Applied Biosystems).

Protein extraction and analysis. Protein was extracted from PDA-cellophane-grown mycelium as detailed in Parlsey et al. (40). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and immunodetection were performed as described in Sambrook and Russell (48). For detection of PGP-1, 20 μg of protein extract was loaded per well, whereas 30 μg of protein extract was loaded for detection of CPGB-1 or S-tagged CPRGS-1. Anti-CPG-1 antibodies (20) were used at a dilution of 1:2,500, and anti-CPGB-1 antibodies (29) were used at a dilution of 1:400. The S-tag HRP Lumiblot kit (Novagen/EMD Biosciences, San Diego, Calif.) was used according to the instructions provided with the kit.

Phylogenetic analysis. The closest homologs of the RGs sequence from C. parasitica (CPRGS-1) were identified by a BLASTP search of genomic sequences from five other ascomycetes and three basidiomycetes. The fungal species names are listed below, each followed by a possible synonym (when available). GenBank Taxonomy ID number, and GenBank accession number or the name of the RGs protein homolog, if known. The protein sequence of the CPRGS-1 homolog from Coprinus cinereus (syn. Coprinus cinereus) (no. 5346) was derived from the nucleotide sequence obtained by a BLASTN search of the database at the Center for Genome Research at the Broad Institute (Cambridge, Mass.). Protein sequences of RGs homologs from Emericella nidulans (syn. Aspergillus nidulans; no. 162425; FlbA) (33), Gibberella zeae (no. 5518; XP_386404), N. crassa (no. 5141; XP_329025), Saccharomyces cerevisiae (no. 4932; Snf2p) (13), Schizosaccharomyces pombe (no. 5534; Rgs1p) (52), S. commune (no. 5334; Thn1) (18), and Ustilago maydis (no. 5270; XP_399719) were obtained from the GenBank database. The sequences were aligned by using CLUSTALX ver. 1.81 (51) with the following alignment parameters: gap opening of 10 and gap extension of 0.20. From this alignment, a data set for phylogenetic analysis was constructed that excluded large insertions and deletions (indels). From C. parasitica, the following RGs residues shown in Fig. 1 were included in the phylogenetic data set: positions 215 to 399, 415 to 491, 579 to 617, 636 to 683, and 712 to 760. (The multiple sequence alignment in Fig. 1 for the three taxa is slightly different than the one obtained for nine taxa, which is not shown.) The total length of the nine sequences varied from 481 residues in Schizosaccharomyces pombe to 763 residues in Ustilago sp. The final data set—large indel regions excluded—contained 389 characters. Approximately 2% of total sequence data still represented indels, and these were coded as completely ambiguous. Unweighted parsimony analysis of amino acid sequences was conducted with PAUP4.0 software (D. L. Swofford; available from Sinauer Associates, Sunderland, Mass.). Protein constists of heuristic searches with TBR branch swapping and 100 random sequence addition replicates. Nonparametric bootstrap analysis (17) of 10,000 pseudoreplicate data sets was performed in a manner identical to that used for the parsimony analysis of the original data set. Bremer support values (3) were calculated by using TreeRot software (M. D. Sorenson, TreeRot, version 2c; Boston University, Boston, Mass.). Average distances across clades were manually calculated by using the table of pairwise difference values available with PAUP4.0. Branch lengths were calculated by using MacClade 4 software (D. R. Maddison and W. P. Maddison; available from Sinauer Associates).

RESULTS

Cloning and sequence analysis of cprgs-1. RGS proteins play an essential role in regulating signal transduction through heterotrimeric G-protein pathways. A C. parasitica RGS homolog
FIG. 2. Phylogenetic analysis under the parsimony criterion of RGS protein sequences from nine taxa within Ascomycota and Basidiomycota. Taxa are referred to by their Linnaean names (see Materials and Methods for synonyms and gene names). Those genera followed by asterisks were also sampled in Bruns et al. (4). Number of most-parasimonious trees: 1; most-parasimonious tree length: 1006; number of parsimony-informative characters: 221; total number of included characters: 389; consistency index: 0.9324; retention index: 0.8265. Bootstrap percentages followed by Bremer support/decay indices are shown above the internal branches. The percent uncorrected average pairwise amino acid differences are shown below branches and in the enclosed box. Branch lengths are proportional to the number of amino acid changes and are optimized on a topology rooted between Ascomycota and Basidiomycota.

was isolated via a PCR-based approach with degenerate PCR primers that were derived from conserved amino acid sequences of several characterized fungal RGS-protein-encoding genes (18, 33). The resulting PCR fragment was sequenced and found to be homologous to FlbA and was subsequently used to screen cDNA and genomic libraries. Sequence analysis of a full-length 3.2-kb cDNA clone revealed several open reading frames. The longest open reading frame, spanning 1,533 nucleotides, encoded the RGS protein and was termed CPRGS-1. The predicted size of CPRGS-1 is 57 kDa, and the amino acid sequence contains the signature sequence of RGS proteins: the RGS domain, as well as two DEP domains.

These nine taxa, the much greater branch lengths of both Hemiascomycotina taxa is very likely a real observation. This indicates that their rate of RGS sequence evolution is accelerated relative to that in Euascomycotina and Basidiomycota. Phenotypic characterization of cprgs-1 deletion mutants. To examine the effect of deletion of the cprgs-1 gene, a knockout construct was made by replacing a 1,030-bp SphI/SmaI fragment within the cprgs-1 open reading frame in pRGSX with a gene cassette that confers hygromycin resistance. The plasmid was digested with KpnI and NotI to release the insert from the vector and transformed into wild-type strain EP155. Of 50 independent transformants, 5 colonies showed an altered phenotype. PCR and genomic Southern blot analysis showed that these three transformants contained one copy of the knockout construct, integrated at the cprgs-1 locus (results not shown). None of these three transformants had vector sequences integrated into the genome (not shown).

Since RGS proteins function to enhance the intrinsic GTPase activity of Gα, deletion of an RGS gene would result in a prolonged activity of its Gα subunit counterpart. Thus, the phenotypic effects of an RGS-null mutation are predicted to be similar to, but perhaps not as severe as, those caused by the presence of a constitutively active Gα allele. As shown in Fig. 3, the phenotype of a Δcprgs-1 strain mimicked that of a strain which contains a copy of a mutationally activated cpg-1 allele, QL1 (49). Both mutations resulted in colonies that showed a complete absence of sporulation and pigmentation and a reduction in radial growth in vitro on solid medium. The mutations also resulted in complete loss of virulence on dormant chestnut stems (not shown). The growth characteristics of the Δcprgs-1 and QL1 strains under conditions of stress were also remarkably similar. Radial growth was reduced under conditions of osmotic stress caused by the addition of KCl or sorbitol to the media and during growth at elevated temperature (34°C). In contrast, growth of both mutant strains on medium-containing medium, which causes oxidative stress, resulted in faster radial growth, compared to standard conditions (Fig. 4 in reference 49 and results not shown).
When cprgs-1 deletion mutant Δcprgs-1-H8 was transformed with the cprgs-1 genomic clone pRGSX, the wild-type phenotype was restored, indicating that the mutant phenotype was caused by specific deletion of RGS, and that the genomic clone pRGSX contains the sequences necessary for correct ectopic expression of the cprgs-1 gene (not shown). Insertion of an S-tag (31) in pRGSX, at the N terminus of the predicted open reading frame, also complemented the gene deletion mutation, indicating that the S-tag does not interfere with RGS function (results not shown).

Deletion of cprgs-1 affects accumulation of G-protein subunits and expression of cryparin. We have previously shown that mutations in or deletions of G-protein subunits affect the stability of the predicted interacting subunits, resulting in reduced accumulation (29, 40, 49). This effect was posttranscriptional, since transcript levels of the corresponding genes remained unaltered. Since RGS proteins interact with Ga subunits, we determined the effect of cprgs-1-deletion on the protein and transcript accumulation of cpg-1 and cpgb-1, which encode Ga and Gb subunits, respectively. Western blot analysis showed that the protein levels of both CPGB-1 and CPGBB-1 were dramatically reduced in the Δcprgs-1 strains (Fig. 4A and B). The level of reduction of CPGB-1 in Δcprgs-1 strains was comparable to that exhibited by the QL1 strain (Fig. 4B).

Transcript levels were analyzed by real-time reverse transcription-PCR (real-time RT-PCR) (Fig. 4C). The absence of cprgs-1 transcript in the Δcprgs-1 strain confirmed deletion of the cprgs-1 gene. Interestingly, the levels of cprgs-1 transcript were increased in the QL1 strain. The cpg-1 transcript levels, which were increased in the QL1 strain as shown before (49), were unchanged in the Δcprgs-1 strains. Transcript levels of cpgb-1 were slightly reduced (<2-fold) in the Δcprgs-1 strains but did not change in the QL1 strain compared to the wild-type strain EP155. The transcript levels of cryparin, which were previously shown by Northern blot analysis to be significantly reduced in activated CPG-1 strains (49), were also nearly undetectable in the Δcprgs-1 strains.

Overexpression of cprgs-1 results in hypersporulation. Whereas deletion of an RGS-encoding gene is predicted to result in constitutive activation of its Gα partner, overexpression of rgs could result in reduced activity of Gα-mediated signaling. To study the effects of overexpression of cprgs-1, the cprgs-1 open reading frame containing a 5′ S-tag was cloned into expression vector pCPX-NBn2 (see Materials and Methods) and introduced into wild-type strain EP155. The phenotype of the colonies ranged from wild type-like to colonies that were reduced in radial growth and pigmentation but exhibited high levels of sporulation (Fig. 5). The N-terminal S-tag allowed detection of the tagged CPRGS-1 protein. Western blot analysis showed accumulation of a protein of ~70 kDa in the transformants but not in wild-type strain EP155 (Fig. 6A).

The hypersporulating strains RSO1 and RSO21, termed RSO for “RGS S-tag overexpressing,” were chosen for further analysis, since Western blot analysis showed that these strains...
accumulated the highest levels of S-tagged RGS protein. These strains also showed a twofold increase in cprgs-1 transcript accumulation (Fig. 6D). The onset of sporulation in the hyper-sporulating strains did not change since the colonies started sporulating ca. 6 days after inoculation, similar to the wild-type strain EP155. No sporulation was observed when RSO strains were cultured in liquid medium, which suppresses sporulation in wild-type EP155. The CPG-1 levels were not changed in these cprgs-1-overexpressing strains (Fig. 6B), whereas the levels of CPGB-1 were slightly reduced (Fig. 6C). Analysis of transcript accumulation by real-time RT-PCR revealed that cryparin gene expression was not affected by overexpression of cprgs-1. The cpg-1 transcript levels were unaltered, whereas the transcript levels of cpgb-1 were reduced in the RSO strains (Fig. 6D).

**Mutational activation of Gα subunits CPG-2 and CPG-3 causes phenotypic changes different from those caused by cprgs-1 deletion.** Although the experimental results described above suggest an interaction between CPRGS-1 and the Gα subunit cpg-1, we extended the study to include cpg-2 and the recently identified third Gα subunit cpg-3 (40). The conserved Gln-204 in one of the GTP-binding domains of CPG-1 is also conserved in CPG-2 (Q-207) and CPG-3 (Q-204) (30). To generate constitutively active alleles, point mutations were made in cDNA clones of cpg-2 and cpg-3 to change Gln-207 in CPG-2 and Gln-204 in CPG-3 to Leu. The mutated cDNAs were cloned into a pCPX expression vector (8; see also Materials and Methods) containing a selectable marker for either benomyl resistance (cpg-2) or hygromycin resistance (cpg-3) resulting in plasmids pCPX-C2QL and pCPX-C3QL, respectively. Plasmid pCPX-C2QL was transformed into the Δcpg-2 strain, and plasmid pCPX-C3QL was transformed into EP155.

Mutational activation of CPG-2 resulted in colonies that grew slower than either the recipient Δcpg-2 strain or the wild-type strain EP155 and showed irregular colony margins but were not impaired in pigmentation and conidiation. Trans-
formants expressing CPG-3-QL were suppressed in pigmentation and conidiation. Radial growth was not affected in the CPG-3-QL transformants (Fig. 7). Two independent transformants containing the CPG-2-QL allele and two independent transformants containing the CPG-3-QL allele were chosen for further analysis.

Western blot analysis of CPG-1 protein levels revealed that they were reduced in strain CPG-2-QL 10, but the levels in the other CPG-2-QL strain and both CPG-3-QL strains were not affected (Fig. 8A). The protein levels of CPGB-1 were reduced in the CPG-2-QL strains but remained unchanged in CPG-3-QL strains (Fig. 8B). The mutants were also tested for accumulation of mRNAs for the genes cprgs-1, cryparin, cpg-1, and cpgb-1 (Fig. 8C). cprgs-1 transcript levels did not change appreciably in the CPG-2-QL and CPG-3-QL strains, whereas the QL1 strain accumulated ~6-fold more cprgs-1 transcript (Fig. 4C). In contrast to the QL1 strain, which expresses barely detectable levels of cryparin transcript, the levels of cryparin transcript were somewhat elevated in the activated CPG-2-QL and CPG-3-QL strains. The transcript levels of cpg-1 and cpgb-1 in the mutant strains were comparable to those in wild-type strain EP155.

**DISCUSSION**

Heterotrimeric G proteins play crucial roles in the regulation of fungal developmental processes, as well as pathogenicity (2). Tight regulation of G-protein-mediated signaling is essential, since both null and activating mutations of the same Gα subunit result in debilitating effects on fungal growth, reproduction, and pathogenicity (15, 19, 27, 35, 45, 56, 58–60). RGS proteins provide an essential function as negative regulators, since they enhance the intrinsic GTPase activity of the Gα subunit, which is a critical step in the deactivation of the cellular response. A large number of mammalian RGS proteins have been isolated and shown to play roles in various signaling pathways (26). Molecular and functional characterization of fungal RGS homologs has been limited to FlbA (33, 58) and the recently identified RgsA (23) in *A. nidulans* and Sst2 in *S. cerevisiae* (reviewed in reference 32). The characterization of CPRGS-1 described here establishes a role for a RGS protein in the modulation of virulence, conidiation, and hydrophobin gene transcription in a plant pathogenic fungus.

*C. parasitica* CPRGS-1 shows an organization similar to several RGS proteins from other lower eukaryotes, such as FlbA (*A. nidulans*), Thn1 (*S. commune*), Sst2 in *S. cerevisiae* (reviewed in reference 32). The characterization of CPRGS-1 described here establishes a role for a RGS protein in the modulation of virulence, conidiation, and hydrophobin gene transcription in a plant pathogenic fungus.
membrane localization (1, 25, 36, 41). Furthermore, studies with a S. cerevisiae strain that constitutively expressed the N-terminal DEP domain of Sst2 showed that the DEP domain is involved in regulating the expression of stress-responsive genes through interaction with other proteins (5). Further studies are necessary to determine the function of the N-terminal DEP domains of CPRGS-1.

Phylogenetic analysis of RGS sequences from nine taxa, all within Ascomycota and Basidiomycota, has yielded a fairly robust result (Fig. 2). Significantly, the relationships are congruent for the six genera cosampled in a previous 18S ribosomal study (see Fig. 4B in reference 4) and with morphology generally (50), indicating that the RGS sequences are likely to be orthologous and that both RGS and 18S gene trees are tracking the species relationships. In the 18S study (4), monophyly of Hemiascomycotina was sensitive to the method of analysis and inclusion of outgroups, reinforcing our cautionary statement (see Results) about the large distances among RGS sequences for these same taxa.

It is particularly interesting that 18S sequences from S. cerevisiae and S. pombe are not long branched, in contrast to their RGS counterparts (4). Although requiring data from additional genes, we could hypothesize that this difference means that the taxa themselves are not undergoing accelerated evolution; rather, acceleration is restricted to a subset of genes. A study by Böller (2) provides suggestive evidence that the Go subunits Gpa1p from S. cerevisiae and S. pombe are also long branched. Since these Go subunits interact with the RGS proteins Sst2 and Rgs1, respectively, we could further hypothesize that multiple sequences within the G-protein signaling pathway have undergone accelerated evolution, perhaps in a coevolutionary sense (see, for example, reference 42). A clearer picture of the evolution of RGS and other molecules within the G-protein signaling pathway will require functional studies from additional species within Hemiascomycotina (and closely related groups), followed by their placement within a phylogenetic context.

It is noteworthy that, despite 69% sequence identity between CPRGS-1 and FlbA from A. nidulans, the respective knockout phenotypes were very different. This follows a trend which has also been observed for Go subunits, where mutations or deletions of homologous Go subunits in closely related fungi often cause vastly different effects, as discussed by Dawe and Nuss (11). Deletion of cprgs-1 resulted in reduced growth, aerial mycelium production, and absence of conidiogenesis, whereas flbA deletion resulted in a so-called fluffy phenotype, characterized by abundant aerial mycelium production, which autolyses as the colony matures and reduced conidiogenesis (33). Inactivation of the RGS-encoding gene thnl in S. communes resulted in phenotypic changes that were similar to deletion of cprgs-1 in C. parasitica. Thnl deletion mutants lacked aerial mycelium, and dikaryons that were homozygous for the thnl mutation did not produce fruiting bodies (44).

Overexpression of both cprgs-1 and flbA (33) caused hyper sporulation in C. parasitica and A. nidulans, respectively. However, whereas the flbA-overexpressing strain produced conidia in liquid culture, which represses sporulation of a wild-type strain, the cprgs-1 overexpressing mutants RS01 and RS021 did not sporulate during growth in liquid culture. The hyper sporulating phenotype of the cprgs-1 overexpressing mutants is probably not caused through inactivation of CPG-1, since Δcpg-1 strains do not conidiate, but rather through interaction with another protein target.

A function for FlbA and Thnl as RGS proteins modulating Go subunit-mediated signaling was indicated by the observation that null mutations in these genes resembled the phenotypes caused by constitutive activation of Go subunit FadA in A. nidulans (58) and of Go subunits ScGP-A and ScGP-C in S. commune (55). Deletion of cprgs-1 resulted in a phenotype that was remarkably similar to that obtained by introduction of a mutationally activated cpg-1 allele, Q1L (49). Furthermore, the response of Δcprgs-1 to chronic heat, hyperosmolarity, and oxidative stress was similar to that of CPG-1-QL strains (reference 49 and results not shown), and neither CPG-1-QL nor Δcprgs-1 was able to infect chestnut stems (results not shown), suggesting that CPRGS-1 mainly interacts with CPG-1. In contrast, constitutive activation of Go subunit CPG-2 did not cause loss of pigmentation or conidiogenesis. In comparison, constitutive activation of Go subunit CPG-3 gave rise to colonies that were in some respects similar to CPG-1-QL mutants but were not impaired in growth and produced abundant aerial mycelia which collapsed in the center of the colony. Interestingly, the phenotype observed for CPG-3-QL mutants was similar to that of the Gβ subunit cpgb-1 deletion mutant (28), although CPGB-1 levels in the CPG-3-QL strains were unaltered. This observation suggests that CPG-3 interacts with CPGB-1 to regulate pigmentation and conidiogenesis and warrants further investigation.

Further evidence for an interaction between CPRGS-1 and CPG-1 was obtained by real-time RT-PCR analysis of cryparin gene expression. Deletion of cprgs-1 or constitutive activation of cpg-1 both resulted in a severe reduction in cryparin transcript levels. In contrast, expression of cryparin was slightly increased in the strains containing cpg-2-QL and cpg-3-QL alleles. Interestingly, S. commune thn1 deletion mutants were shown to be deficient in expression of Sc3 (54), a hydrophobin-encoding gene that is abundantly expressed during formation of aerial hyphae (37). Further evidence is needed to determine whether a common regulatory pathway for hydrophobin gene expression, regulated through RGS proteins and Go subunits, exists in filamentous fungi.

As we have noted in earlier studies, mutations in G-protein subunits can have dramatic effects on posttranscriptional accumulation of their presumptive G-protein partners (28, 40, 49). This observation was recently confirmed in N. crassa (57), indicating the existence of a regulatory pathway common to other fungi and possibly other organisms. In the present study we have shown that deletion of the gene encoding CPRGS-1, which is predicted to interact with Go subunit CPG-1, also results in severe posttranscriptional reduction of CPG-1 levels and CPGB-1 levels. These observations suggest that G-protein subunit stability and degradation provides an additional level of control on G-protein-mediated signaling and reinforces the fact that the effects of a posttranscriptional control mechanism must be considered when results of mutations in signaling components are interpreted.

In addition, we observed an effect of CPG-1 on the transcription of cprgs-1. cprgs-1 transcript levels increased dramatically in response to constitutive activation of CPG-1, suggesting the existence of a control mechanism on the transcriptional
level to regulate activity of the CPG-1-mediated signaling pathway. This effect was not observed in CPG-2-QL or CPG-3-QL strains, providing additional support for a specific interaction between CPRGS-1 and CPG-1.

Although CPG-1 protein levels were severely reduced in Δcprgs-1 strains, the changes observed in cprgs-1 deletion strains are likely to be mediated through the CPG-1 signaling pathway. CPG-1-QL has a strong, dominant effect, since transformation of a cpg-I-QL expressing plasmid into a wild-type, Δcpgb-1, or Δcpg-1 strain resulted in the same phenotype, regardless of the genetic background of the recipient strain (49). Although interaction of CPRGS-1 with CPG-2 or CPG-3 cannot be ruled out, it is likely that C. parasitica expresses several RGS-encoding genes, each with specificity to a particular Gα subunit. For example, S. cerevisiae has two RGS encoding genes, Sst2 and Rgs2, which regulate activity of Gα subunits Gpa1 and Gpa2, respectively. These two RGS proteins share little homology outside their RGS domains and have nonoverlapping specificity for their respective Gα subunits (52). It will be of interest to identify and characterize additional RGS proteins in C. parasitica and determine the specificities of each RGS protein for the various Gα subunits and their corresponding regulatory pathways.

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