Microarray analysis of *Cryphonectria parasitica* G α - and G $\beta\gamma$ -signalling pathways reveals extensive modulation by hypovirus infection

Angus L. Dawe,*† Gert C. Segers,* Todd D. Allen, Vanessa C. McMains‡ and Donald L. Nuss

Correspondence

Donald L. Nuss
nuss@umbi.umd.edu

Center for Biosystems Research, University of Maryland Biotechnology Institute, 5115 Plant Sciences Building 036, College Park, MD 20742, USA

Using an established spotted cDNA microarray platform, the nature of changes in the transcriptional profiles of 2200 unique genes from the chestnut blight fungus *Cryphonectria parasitica* in response to the absence of either the G α subunit CPG-1 or the G β subunit CPGB-1 has been explored. It is reported that 216 transcripts were altered in accumulation in the $\Delta cpg-1$ strain and 163 in the $\Delta cpgb-1$ strain, with a considerable overlap (100 genes) that were changed in both cases. Of note, these commonly altered transcripts were changed in the same direction in every instance, thus suggesting a considerable redundancy in pathway control or extensive crosstalk. To further knowledge of the potential impact on G-protein-signalling of infection by hypovirus CHV1-EP713, the accumulation of CPG-1 and CPGB-1 was also investigated by Western analysis. It was demonstrated that both signalling components were reduced in abundance to approximately 25 % of wild-type levels, while their transcripts were slightly elevated. Comparison of a list of genes with altered expression in the presence of CHV1-EP713 to the data obtained in the absence of either G-protein subunit showed that more than one-half of all the transcripts changed by hypovirus infection were also changed in at least one G-protein mutant strain, with one-third being changed in both. Significantly, 95 % of the co-changed genes were altered in the same direction. These data provide the first evidence for modulation of G β protein levels as well as the G $\beta\gamma$ -signalling pathways by hypovirus infection, and support the hypothesis that modification of G-protein-signalling via both G α and G $\beta\gamma$ provides for a significant contribution to hypovirus-mediated phenotype.

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INTRODUCTION

Heterotrimeric GTP-binding proteins (G-proteins), consisting of three subunits, G α , G β and G γ , are ubiquitous signalling components that function to enable eukaryotic organisms to react to environmental stimuli (reviewed by Hamm, 1998). Upon activation, G α dissociates from the G $\beta\gamma$ complex, allowing subsequent regulation of two downstream pathways. Multiple G-protein subunits have been isolated from filamentous fungi, and previous studies have demonstrated the requirement for a functional G-protein-mediated signalling cascade for fungal growth, reproduction and virulence (reviewed by Bölker, 1998;

Lengeler *et al.*, 2000). However, the precise roles of specific subunits, their effectors and the nature of extracellular signals that trigger a given response, are still poorly understood.

Cryphonectria parasitica, the chestnut blight fungus, is the plant pathogen responsible for the destruction of the native populations of American chestnut throughout the natural range of this tree, beginning in the early 20th century (Merkel, 1906). A relationship between G-protein-signalling pathways and virulence of *C. parasitica* has been suggested by the deletion of the genes encoding two G α subunits (*cpg-1* and *cpg-2*). Absence of CPG-1 results in an avirulent strain that is also defective in asexual sporulation and pigment production and is reduced in growth, while a deficiency in CPG-2 leads to only minor alterations in phenotype from wild-type and little change in virulence (Gao & Nuss, 1996). A G β subunit, CPGB-1, has also been identified in this organism (Kasahara & Nuss, 1997) and the $\Delta cpgb-1$ strain is similar to $\Delta cpg-1$ in that it is minimally virulent with no pigmentation and greatly reduced sporulation. However, in

*These authors contributed equally to this paper.

†Present address: Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA.

‡Present address: Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health Bldg 50, Bethesda, MD 20892-2715, USA.

Abbreviation: G-protein, heterotrimeric GTP-binding protein.

contrast to the phenotype of the G α knockout, the lack of CPGB-1 results in increased biomass production (Kasahara & Nuss, 1997). Although precise functions have not yet been determined for the G-protein subunits identified in *C. parasitica*, the disruption of signalling caused by the absence of CPG-1 or CPGB-1 mimics some of the phenotypic changes induced by infection with members of the virulence-attenuating virus family *Hypoviridae* that occur in natural populations of *C. parasitica*. Specifically, the prototypic hypovirus CHV1-EP713 has been shown to reduce growth (in common with Δ cpg-1) as well as asexual sporulation, pigmentation and virulence (in common with Δ cpg-1 and Δ cpgb-1 (reviewed by Dawe & Nuss, 2001).

Chen *et al.* (1996) demonstrated that a potentially large number of changes in transcript accumulation occurred in response to hypovirus infection of *C. parasitica* by using mRNA differential display technology. During this study the authors also noted a connection between G-protein-mediated cAMP levels and aspects of the hypovirulent phenotype. Further analysis of the effect of hypovirus infection has been facilitated by the development of a robust spotted cDNA microarray platform (Allen *et al.*, 2003) based on approximately 2200 unique ORFs previously identified by expressed sequence tags (Dawe *et al.*, 2003). Allen *et al.* (2003) examined the transcriptional changes of this population of genes in the presence of hypovirus compared to the expression levels in wild-type (uninfected) mycelium, concluding that significant and persistent reprogramming of the *C. parasitica* transcriptome occurred in the presence of CHV1-EP713. A comparison of the effects on transcript accumulation of CHV1-EP713 and CHV1-Euro7, a naturally occurring hypovirus that is in the same family but produces less severe symptoms on fungal phenotype, has demonstrated the utility of comparing multiple datasets from this microarray system (Allen & Nuss, 2004).

Therefore, as part of a continuing effort to understand the role of G-protein-signalling in the growth, reproduction and virulence of this plant pathogen, we have expanded our use of the microarray platform to examine the effect of the absence of CPG-1 or CPGB-1 on global transcriptional profiles. We note striking similarities and overlap between those genes whose transcripts are altered in abundance when either G-protein component is lacking. In an attempt to better understand the relationship between G-protein-mediated signalling and hypovirus infection, we have used antibodies to the G α and G β subunits to quantify a reduction in the accumulation of both proteins in the presence of hypovirus. Subsequently, we also compared the above microarray data to those obtained by examining hypovirus-infected mycelium and noted additional significant overlap between the datasets. The observed transcriptional profiles point to the modulation of G-protein-signalling pathways as a significant contributor to the phenotype of hypovirus-infected mycelium. Implications for the modulation of specific genes are discussed in the context of fungal development and virulence.

METHODS

Fungal strains and growth conditions. The strains of *Cryptoglyphus parasitica* used in this study were EP155 (ATCC 38755), the isogenic strain EP155/CHV1-EP713 (ATCC 52571) that is infected with the prototypic hypovirus CHV1-EP713 (Choi & Nuss, 1992), Δ cpg-1 (Gao & Nuss, 1996) and Δ cpgb-1 (Kasahara & Nuss, 1997). All strains were maintained on solid medium [3.9% (w/v) Difco potato glucose agar (PDA; Becton Dickinson)] at a temperature between 22 and 24 °C with a 12 h light/dark cycle at 1300–1600 lx. Cultures used for protein or RNA preparations were grown under similar conditions on cellophane membranes overlaying PDA.

Extract preparations and Western blotting. Total protein extracts for Western blotting were prepared using the method of Parsley *et al.* (2003). Dilution immunoblot quantification of relative G-protein subunit levels was performed as described previously using antisera raised against purified CPG-1 and CPGB-1 that specifically recognizes these proteins (Parsley *et al.*, 2003). Total RNA extracts were prepared as described by Allen *et al.* (2003).

Microarray slide printing. A second-generation spotted cDNA chip was constructed from the same *C. parasitica* EST library printing plates used to construct the first-generation chips profiled in Allen *et al.* (2003). The EST library represented approximately 2200 independent transcripts as determined by single pass sequences and contig analysis (Dawe *et al.*, 2003). For the second print-run, GAPS II-coated slides from Corning (coated with γ -amino-propyl-silane) were used. Purified PCR products were singly arrayed with a mean spot diameter of 100 μ m and a spot spacing of 300 μ m. Following printing, the spotted cDNA was cross-linked, washed and blocked as described by Allen *et al.* (2003).

Fluorescent probe generation, hybridization and scanning. Fluorescence-labelled cDNA probes were prepared from total RNA (25 μ g per probe) by direct incorporation of Cy3- or Cy5-dUTP using the CyScribe First-Strand cDNA Labelling Kit (Amersham Pharmacia) primed with oligo(dT) according to the manufacturer's instructions. Unincorporated nucleotides were removed using a Microcon-30 spin column and processed according to Allen *et al.* (2003). Pre-hybridization, hybridization and post-hybridization wash steps were as suggested by the manufacturer of GAPS II slides (Corning). Each hybridized chip was scanned in both Cy3 and Cy5 channels with an Affymetrix 418 Scanner as described by Allen *et al.* (2003).

Microarray data management, processing and analysis. Scanned images were interpreted with Spotfinder (version 1.0; <http://www.tigr.org>). Raw data files are available as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>. Further data processing and analyses were as described by Allen *et al.* (2003). Briefly, normalization of raw fluorescent signal was achieved by utilizing algorithms present in MIDAS software (<http://www.tigr.org/software>). Dye-bias effects were corrected utilizing LOWESS, a local linear regression algorithm, while differences between repeated hybridizations were managed by standard deviation regularization, i.e. by rescaling each spot's cy3 and cy5 measurement using the standard deviation of cy3 and cy5 values as calculated across all experiments. Differentially expressed genes were identified as spots with log₂ ratios equal to or greater than ± 2 standard deviations from the mean log₂ ratio of each dataset in a minimum of three of four hybridizations. To ensure that independent lists of differentially expressed genes reflected biological differences rather than technical differences associated with different print runs, the hybridizations described by Allen *et al.* (2003) between hypovirus-free *C. parasitica* strain (EP155) and *C. parasitica* infected with the prototypic hypovirus CHV1-EP713 were repeated. Therefore, all profiling results reported here are from hybridizations performed on

the same set of second generation chips. Where indicated, mean fold change data were derived from $\log_2$ data, such that fold change = $2^{\text{mean } \log_2 \text{ ratio}}$. Comparisons between lists and curation of differentially expressed clone data were performed as described by Allen & Nuss (2004). Minor refinement of the non-redundant lists was achieved by re-examining any clones whose BLASTX hit *E*-value was reported by Dawe *et al.* (2003) as greater than 1×10^{-10} . Any such clones were verified for redundancy within the library population using BLASTN results obtained by comparing the library to itself, thus eliminating clones that had different, but weak, BLASTX hits yet were in fact derived from the same mRNA sequence. Complete redundant data tables are available as supplementary data with the online version of this paper at <http://mic.sgmjournals.org>

Real-time RT-PCR validation of microarray data. Probes and oligonucleotides for the particular clones to be tested were obtained from Integrated DNA Technologies (Coralville). Each clone to be verified was tested using RNA from two separate preparations, in triplicate on each occasion, using an Applied Biosystems GeneAMP 5700 sequence detection system and an Applied Biosystems TaqMan reverse transcription kit, according to the manufacturer's instructions and as described by Allen *et al.* (2003). Validation failure was determined to be those instances where microarray and real-time RT-PCR values indicated opposite effects on transcription of the tested transcript in the same strain with respect to wild-type values

RESULTS

Similar transcriptional profiles in strains deleted for *cpg-1* or *cpgb-1*

Previous studies have shown the interdependence of protein levels of the G-protein subunits CPG-1 and CPGB-1, since the absence of either subunit caused a reduction in the accumulation of the other (Parsley *et al.*, 2003). Additionally, this was specific to CPG-1: the absence of a second $G\alpha$ subunit (CPG-2) did not affect the amount of CPGB-1 present, suggesting that CPG-1 and CPGB-1 represent cognate functional partners (Parsley *et al.*, 2003). With the advent of a robust spotted cDNA microarray system for this organism (Allen *et al.*, 2003) derived from expressed sequence tags (Dawe *et al.*, 2003), we explored the potential functional relationship between CPG-1 and CPGB-1 by examining transcriptional changes wrought by the deletion of the respective genes.

Microarray analysis revealed changes deemed to be significant in 416 clones, representing 216 different transcripts (116 downregulated, 100 upregulated) when the $\Delta cpg-1$ strain was compared to the isogenic wild-type strain EP155 (supplementary data table A). When the $\Delta cpgb-1$ strain was similarly compared to EP155, 300 clones, representing 163 transcripts (69 downregulated, 94 upregulated) were altered in abundance (supplementary data table B). Comparison of the two non-redundant lists of altered transcripts showed considerable overlap (Fig. 1a). One hundred transcripts were found to be changed in both strains, representing 46.3% of the total for $\Delta cpg-1$ and 61.3% of the total for $\Delta cpgb-1$. Further analysis of these 100 transcripts demonstrated even closer similarity, since all of these changes were coordinately regulated in both strains (i.e. a clone upregulated in the absence of CPG-1 was also

upregulated in the absence of CPGB-1; Fig. 1b). As a general trend, the magnitude of change observed for a given transcript in the $\Delta cpg-1$ mutant was greater than that observed in the $\Delta cpgb-1$ strain.

Reduced quantities of G-protein subunits in the presence of hypovirus

Previous studies (Chen *et al.*, 1996; Choi *et al.*, 1995) had suggested a potential link between G-protein-mediated signal transduction and the attenuation of fungal virulence as a result of infection with a member of the genus *Hypovirus*, CHV1-EP713. Choi *et al.* (1995) used antiserum raised against a peptide derived from the CPG-1 sequence and observed a greatly reduced quantity of an immune-reactive protein species in the presence of hypovirus. To provide specific information on changes in the accumulation of CPG-1 or CPGB-1, we applied a dilution immunoblot method coupled with antisera raised against full-length purified CPG-1 and CPGB-1 proteins that had been used successfully in previous studies (Parsley *et al.*, 2003). This enabled assessment of the abundance of both the α subunit, CPG-1, and the β subunit, CPGB-1, in hypovirus-infected mycelium relative to wild-type (Fig. 2a and b). Both G-protein subunits were found to be decreased in abundance to 25% of the wild-type level reflected by the altered end points of 0.63–2.5 μ g for CPG-1 and 1.25–5.0 μ g for CPGB-1, providing the first reported evidence of altered accumulation of a $G\beta$ subunit in response to hypovirus infection. Furthermore, these changes were determined to be the result of post-transcriptional events. Real-time RT-PCR measurement of the relative transcript abundance suggested that both *cpg-1* and *cpgb-1* were elevated in the presence of hypovirus, since the values obtained were greater than 1.0 when referenced to wild-type data (Fig. 2c).

Expression profile similarity between G-protein mutants and hypovirus infected mycelium

To further examine the potential effects of hypovirus infection upon G-protein-modulated pathways and their targets, we compared the analysis of both G-protein deletion mutants to hypovirus-infected mycelium. Microarray data were obtained using RNA preparations from EP155 infected with hypovirus CHV1-EP713 and referenced against values for uninfected EP155. Fig. 3(a) shows a hierarchical clustering view of the entire redundant dataset from each of the three strains. Since similarly altered transcripts are clustered together in this analysis, the convergence of the coloration in each experiment suggests similarly altered transcriptional profiles, i.e. genes differentially expressed in one instance generally behaved the same way in the other two experimental conditions.

Condensing the hypovirus-infected data to include only the information for individual genes (a non-redundant list) showed that 136 different transcripts (62 downregulated, 74 upregulated) were identified as differentially expressed

Fig. 1. Similarity in expression profiles for two G-protein mutants. (a) Venn diagram showing overlap of expression profiles for $\Delta cpg-1$ and $\Delta cpgb-1$. (b) Bar chart illustrating the magnitude of change in transcript levels predicted by microarray for the 100 similarly expressed genes from $\Delta cpg-1$ (red bars) and $\Delta cpgb-1$ (blue bars).

(from 232 clones described in supplementary data table C). As noted in Methods, differential expression was defined as genes with $\log_2$ ratios ≥ 2 standard deviations from the mean $\log_2$ ratio of each dataset in a minimum of three out of four hybridizations. This allowed analysis of the overlap between the lists of changed transcripts from both G-protein mutants (illustrated by the Venn diagram in Fig. 3b). Seven different classes of gene expression changes were noted, representing those changed in just one, a pair or all three of the strains examined. Combining the data for classes AV (changed in $\Delta cpg-1$ and EP155/CHV1-EP713), BV (changed in $\Delta cpgb-1$ and EP155/CHV1-EP713) and ABV (changed in $\Delta cpg-1$, $\Delta cpgb-1$ and EP155/CHV1-EP713), more than half (73 or 53.7 %) of the transcripts found to be altered in the presence of hypovirus were also changed in at least one of the G-protein-deficient strains. Of these, 95 % (69 genes) demonstrated coordinate regulation in each condition.

Class ABV comprised 45 transcripts, representing almost one-third (33.1 %) of the total genes altered by hypovirus infection, that were also found to be altered in both of the G-protein mutants (Table 1). Analysis of this class using hierarchical clustering (Fig. 3c) illustrated that 43 transcripts (96 %) were coordinately changed across all three samples. The two clones that contradicted this behaviour were upregulated in both G-protein mutants, but downregulated in the presence of CHV1-EP713. Additionally, the magnitude (expressed as the mean fold change, where $\text{fold change} = 2^{\text{mean } \log_2 \text{ ratio}}$) of transcriptional changes undergone by these 43 genes were generally lower in

the hypovirus-infected strain than in either the $\Delta cpg-1$ or $\Delta cpgb-1$ mutant as indicated by the brightness of colour on the cluster diagram (Fig. 3c) and the values in Table 1.

Validation of microarray predictions by real-time RT-PCR

To confirm the changes indicated by the microarray analyses, four or five different transcripts were chosen from each of the different classes denoted in Fig. 3(b). Probes for real-time RT-PCR were then used to independently assess the relative levels of these transcripts as described in Methods. Table 2 shows that only three clear false-positive results were obtained from 53 different probe/RNA combinations, a rate of less than 6 %. Considering the comparable validation values obtained by Allen *et al.* (2003) and Allen & Nuss (2004), these data provided a high degree of confidence in the transcriptional changes that were projected by microarray analysis. In addition to confirming the changes predicted by microarray analysis, we also examined samples that were reported as unchanged by microarray analysis for the genes listed in Table 2, e.g. genes in class A were tested for changes in $\Delta cpgb-1$ and in EP155/CHV1-EP713. A total of 25 of 36 samples that were scored as negative by microarray for this group of genes showed twofold or greater changes by RT-PCR. Thus, the microarray analysis appears to be underreporting the overlap in changes in transcript accumulation for the three strains examined. Since a significant level of coordinate transcriptional regulation was observed in all three strains for a

Fig. 2. Determination of the relative accumulation of the CPG-1 and CPGB-1 proteins in mycelium infected with CHV1-EP713 compared to wild-type tissue. Total protein loaded per lane (μ g) is noted above each lane and location of molecular mass markers is indicated to the right of each blot. (a) CPG-1 levels detected with antiserum to purified protein. The end point was determined as the least amount of total protein at which a band could be consistently discerned in multiple experiments: 0.63 μ g in EP155, 2.5 μ g in the presence of CHV1-EP713. The upper band represents a cross-reactive species that is neither CPG-1 nor CPG-2, but may represent a third uncharacterized G α , CPG-3, identified by Parsley *et al.* (2003). (b) CPGB-1 levels detected with antiserum to purified protein. The end point was determined as the least amount of total protein at which a band could be consistently discerned in multiple experiments: 1.25 μ g in EP155, 5.0 μ g in the presence of CHV1-EP713. (c) Relative transcript accumulation for *cpg-1* and *cpgb-1* in the EP155/CHV1-EP713 strain determined using real-time RT-PCR and probes specific to these sequences (Parsley *et al.*, 2003). Values from six samples were normalized to EP155 data (horizontal line at 1.0).

different subset of genes (Fig. 3c; Table 2) and the genes examined were all differentially expressed in one or two of the three strains, a greater degree of overlap is not unexpected. Moreover, it is known that microarray technology is prone to underreporting changes in transcript accumulation (Cole *et al.*, 2003). Adjustments to correct for this discrepancy would further emphasize the similarities in expression profile changes resulting from independent disruption of *cpg-1* and *cpgb-1* and infection by hypovirus CHV1-EP713.

DISCUSSION

The importance of G-protein-mediated signalling pathways for the growth, development and pathogenicity of filamentous fungi has been well documented (Bölker, 1998; Lengeler *et al.*, 2000). Identification of components of this system from *C. parasitica* has demonstrated that the G-proteins CPG-1 and CPGB-1 play a crucial role in phenotypic traits such as pigment generation and asexual sporulation as well as pathogenicity (Choi *et al.*, 1995; Kasahara & Nuss, 1997). Furthermore, modulation of intracellular signalling by infection with hypoviruses has been suggested as a contributing mechanism that results in the hypovirulent phenotype by altering host gene expression (Chen *et al.*, 1996). Recent development of a spotted cDNA microarray for *C. parasitica* has enabled simultaneous monitoring of the transcriptional responses of 2200 genes that occur upon infection with the hypovirus CHV1-EP713 (Allen *et al.*, 2003). In the current study, we have expanded the use of this platform to quantify gene expression in the context of mutants deleted for *cpg-1* or *cpgb-1*. This has allowed us to correlate the differences in phenotype with transcriptional profiles to obtain further information on potential downstream targets and assess similarities to those changes observed in the presence of hypovirus.

A comparison of the data obtained from the two G-protein subunit deletion mutants demonstrated significant similarity, despite their superficially divergent phenotypes. The overlap of 100 similarly altered genes, 46.3 % of the total for Δ *cpg-1* and 61.3 % of the total for Δ *cpgb-1*, is likely to represent those targets that are the most sensitive to disruptions of the G-protein pathway. Also, this observation highlights the difficulties of interpreting the functional significance of phenotype based on single-gene knockouts of G-protein subunits or components of G-protein-signalling pathways. It is particularly striking that all of these transcripts are not just altered in abundance in both scenarios, but are also changed in the same direction. A likely explanation for the considerable similarity in the transcriptional profiles of the two G-protein knockout strains can be attributed to a previously observed two- to fourfold reduction in the levels of CPG-1 in the absence of CPGB-1 and vice versa (Parsley *et al.*, 2003). Interestingly, these protein reductions were specific to CPG-1 and CPGB-1; the deletion of a second G α subunit, CPG-2 (Choi *et al.*, 1995) had no effect on the accumulation of

Fig. 3. Similarity in expression profiles for two G-protein mutants and hypovirus-infected mycelium. Strain key for the cluster diagrams: $\Delta\alpha$, $\Delta cpg-1$; $\Delta\beta$, $\Delta cpgb-1$; V, EP155/CHV1-EP713. Colour brightness is proportional to the magnitude of the change with red indicating genes upregulated compared to wild-type, green indicating down-regulation. (a) Hierarchical clustering diagram of the dataset representing all 3864 redundant clones using mean $\log_2$ (cy3/cy5) values from the four total hybridizations. (b) Venn diagram illustrating the number of common clones identified in the non-redundant data for the three strains. Class key: A, changed in $\Delta cpg-1$ only; B, changed in $\Delta cpgb-1$ only; V, changed in EP155/CHV1-EP713 only. Combinations (AB, AV, BV, ABV) refer to overlapping populations. Numbers of clones in each class are indicated in parentheses. (c) Hierarchical clustering diagram of the dataset representing the non-redundant ABV class of transcripts prepared as in (a).

CPGB-1, suggesting that CPGB-1 and CPG-1 may represent cognate functional partners, a hypothesis that our data further support. Alternatively, the profile similarities may reflect a degree of crosstalk between the CPG-1 and CPGB-1-mediated cascades, or a combination of immediate functional interaction and downstream crosstalk. As a general theme, we also noted that the magnitude of transcript changes was larger in the absence of CPG-1 rather than CPGB-1. A specific explanation for this observation is not apparent at this time. However, there could be a correlation between this phenomenon and the severity of the phenotype of the $\Delta cpg-1$ strain since it is significantly more impaired in growth and development than $\Delta cpgb-1$.

Potentially important signalling proteins that may represent candidates responsible for significant contributions to the G-protein mutant phenotypes include a phosducin-like-protein BDM2 (AEST-19-D-04) and the PAS/LOV family member VVD (AEST-27-H-09). Both showed considerable reduction in transcript level in both G-protein deletion strains in comparison to EP155 (supplementary data Tables A and B). BDM2, a homologue of PLP2p from *Saccharomyces cerevisiae* (Flanary *et al.*, 2000) represents a second phosducin-like protein that is also similar to BDM-1 (23 % identical, 36 % similar), a negative regulator of the $G\beta\gamma$ -signalling pathway (Kasahara *et al.*, 2000) whose gene is closely linked to the *cpgb-1* gene in the *C. parasitica* genome (Dawe *et al.*, 2003). Deletion of *bdm-1* resulted in a phenotype identical to that of $\Delta cpgb-1$ (Kasahara *et al.*, 2000) and a large reduction in the amount of CPGB-1 protein compared to wild-type (A. L. Dawe & D. L. Nuss, unpublished observations). Proteins similar to BDM-1 and PLP2 have been identified in *Dictyostelium discoideum* as well as *S. cerevisiae*, and in both cases the BDM-1 homologue appears

to be involved in $G\beta\gamma$ -signalling while deletion of the *bdm2/plp2* gene results in a lethal phenotype (Blaauw *et al.*, 2003; Flanary *et al.*, 2000). It is presently unclear how the function of BDM2/PLP2 might fit into the overall G-protein-signalling scheme of *C. parasitica*, but the reduction of the *bdm2* transcript in the absence of either CPG-1 or CPGB-1 may indicate a connection that would benefit from further study.

In *Neurospora crassa*, the VIVID (VVD) protein serves as a blue-light receptor for photo-adaptation (Schwerdtfeger & Linden, 2003) and contributes to mechanisms that control the regulation of circadian rhythms (Heintzen *et al.*, 2001). Both G-protein mutants are deficient in asexual sporulation and pigmentation (Gao & Nuss, 1996; Kasahara & Nuss, 1997), two characteristics that, in wild-type mycelium, are enhanced by high light intensity. Therefore the observation of a reduction in the level of *vvd* transcript provides for a potential mechanism by which sporulation and pigmentation are affected in the G-protein mutants. Intriguingly, although these same characteristics are also altered in the hypovirus-infected mycelium, the *vvd* transcript was not detected as changed by either microarray or real-time RT-PCR. This presents the possibility that sporulation and pigmentation are affected by a G-protein-independent mechanism in the presence of CHV1-EP713.

Previous studies had implicated the G-protein-signalling pathway as a possible target of hypovirus-mediated alteration that could contribute to the pleiotropic effects observed in infected mycelia (Choi *et al.*, 1995; Gao & Nuss, 1996). In our experiments, we have used antiserum raised against full-length CPG-1 in a method that has been previously determined to provide accurate assessment of

Table 1. Microarray results for the clones in the ABV class (see Fig. 3b) and associated functional identification based on BLASTX analysis

Shaded rows highlight clones that behaved differently in the presence of hypovirus from their values in the G-protein-deficient strains.

Clone no.*	E-value†	BLASTX result†	Magnitude of change		
			$\Delta cpg-1$	$\Delta cpgb-1$	EP155/CHV1-EP713
38-H-09	3×10^{-36}	Glutathione S-transferase [<i>Schizosaccharomyces pombe</i>]	66.12	9.73	5.93
32-C-04	5×10^{-20}	Probable oxidoreductase [<i>Pseudomonas aeruginosa</i> PA01]	52.06	15.57	7.74
21-F-04	—	No significant hit	47.29	23.23	4.23
25-F-08	—	No significant hit	45.68	11.1	5.58
19-A-05	8×10^{-42}	Predicted protein [<i>Neurospora crassa</i>]	43.59	6.57	3.88
11-E-09	4×10^{-11}	Cytochrome P450, putative [<i>Arabidopsis thaliana</i>]	42.56	18.28	4.78
26-G-12	—	No significant hit	41.4	11.53	6.46
24-H-08	2×10^{-18}	O-Methyltransferase [<i>Aspergillus parasiticus</i>]	35.6	77.6	6.15
24-D-10	7×10^{-23}	Oxidoreductase [<i>Clostridium acetobutylicum</i>]	35.28	13.96	5.89
37-C-08	—	No significant hit	32.74	12.43	5.46
27-A-03	—	No significant hit	31.83	9.77	7.24
31-G-11	5×10^{-19}	Aminocyclopropane-1-carboxylate oxidase [<i>Phaseolus vulgaris</i>]	25.21	12.15	6.07
05-B-03	3×10^{-19}	TOXD protein [<i>Cochliobolus carbonum</i>]	21.77	6.97	12.15
37-C-06	6×10^{-42}	Predicted protein [<i>Neurospora crassa</i>]	19.04	4.2	12.61
29-A-07	4×10^{-13}	Chitinase [<i>Beta vulgaris</i>]	18.45	14.18	−2.78
15-F-07	—	No significant hit	13.77	8.07	4.37
06-B-06	1×10^{-38}	Predicted protein [<i>Neurospora crassa</i>]	11.93	7.66	8.47
01-H-03	—	No significant hit	11.16	5.68	5.26
40-B-02	1×10^{-14}	Reductase [<i>Gibberella zeae</i>]	11.1	4.98	4.95
06-A-07	—	No significant hit	10.67	6.8	4.22
28-F-06	7×10^{-48}	Hypothetical protein [<i>Neurospora crassa</i>]	10.12	4.88	10.9
02-A-07	7×10^{-50}	Putative S-adenosyl-L-homocysteine hydrolase; Sah1p [<i>Saccharomyces cerevisiae</i>]	10.08	4.49	4.23
08-F-11	2×10^{-28}	Hypothetical protein [<i>Neurospora crassa</i>]	8.74	4.65	4.61
38-B-07	4×10^{-26}	Dehydrogenase [<i>Bradyrhizobium japonicum</i>]	7.58	5.94	−2.66
21-D-11	—	No significant hit	7.23	6.67	6.78
21-H-05	—	No significant hit	5.4	10.28	5.9
16-D-07	2×10^{-90}	Hypothetical protein [<i>Neurospora crassa</i>]	−4.8	−8.1	−5.27
32-C-06	—	No significant hit	−5.19	−4.79	−6.29
11-D-08	1×10^{-109}	Endothiapepsin [<i>Cryphonectria parasitica</i>]	−5.97	−6.6	−4.69
33-D-02	—	No significant hit	−7.4	−4.17	−11.49
36-C-10	—	No significant hit	−7.5	−7.13	−7.42
27-E-03	—	No significant hit	−7.56	−4.43	−5.83
03-G-06	3×10^{-91}	Acid proteinase eapB [<i>Cryphonectria parasitica</i>]	−8.0	−10.04	−16.9
34-B-07	2×10^{-13}	Putative dTDP-glucose 4,6-dehydratase [<i>Pinus sylvestris</i>]	−8.44	−8.82	−3.94
37-C-12	—	No significant hit	−11.32	−48.51	−31.26
30-C-09	6×10^{-65}	Mst12 [<i>Magnaporthe grisea</i>]	−11.96	−4.66	−5.06
23-B-10	—	No significant hit	−12.92	−12.25	−10.79
18-H-07	5×10^{-41}	Hypothetical protein [<i>Neurospora crassa</i>]	−13.17	−5.0	−6.16
38-C-04	1×10^{-108}	Heat-shock protein 70 (hsp70) [<i>Neurospora crassa</i>]	−14.16	−9.08	−42.6
20-G-02	—	No significant hit	−15.02	−6.37	−5.66
11-E-01	2×10^{-28}	Hypothetical protein [<i>Neurospora crassa</i>]	−15.84	−5.98	−7.42
35-C-02	2×10^{-77}	Nuclease P1 [<i>Penicillium citrinum</i>]	−25.56	−6.43	−4.82
05-F-11	2×10^{-84}	Nuclease PA3 [<i>Penicillium</i> sp.]	−28.98	−8.81	−7.39
09-E-04	4×10^{-45}	Aspergillopepsin II [<i>Aspergillus niger</i>]	−49.54	−36.31	−4.72
32-B-01	—	No significant hit	−76.59	−11.19	−50.2

*Clone number refers to the AEST arrayed designations applied by Allen *et al.* (2003) to the CEST sequences identified by Dawe *et al.* (2003).†BLASTX data refer to the analysis of Dawe *et al.* (2003) with the addition of secondary hit information where the primary hit was uninformative. No functions have been ascribed unless the E-value of the hit was 1×10^{-10} or lower. Values refer to fold changes predicted by microarray when compared to EP155. Positive numbers indicate increased transcript abundance in the tested strain, negative numbers a reduction in abundance.

Table 2. Real-time RT-PCR validation of microarray predictions for transcriptional changes in the *Δcpg-1*, *Δcpgb-1* and EP155/CHV1-EP713 strains

Values listed are the mean of six samples as described in Methods. Shaded boxes highlight those validations that failed. MA, fold change values obtained by microarray; RT, fold change values obtained by real-time RT-PCR.

Class	Clone ID	<i>Δcpg-1</i>	
		MA	RT
A	AEST-09-A-05	−11·36	−14·69
A	AEST-14-H-02	−9·76	−2·99
A	AEST-27-F-10	−9·54	−20·44
A	AEST-28-E-10	−15·09	−25·49
		<i>Δcpgb-1</i>	
		MA	RT
B	AEST-05-C-02	−11·74	−16·39
B	AEST-08-G-08	4·80	14·57
B	AEST-11-A-04	−14·52	−26·97
B	AEST-11-B-12	−5·63	−4·75
		EP155/CHV1-EP713	
		MA	RT
V	AEST-08-F-10	536·67	4·36
V	AEST-10-A-11	−5·89	−2·05
V	AEST-13-C-05	29·23	2·56
V	AEST-31-F-09	15·71	77·26
		<i>Δcpg-1</i>	
		MA	RT
AB	AEST-07-B-12	32·26	−1·16
AB	AEST-11-D-08	−5·21	−12·48
AB	AEST-27-H-09	−8·75	−13·13
AB	AEST-29-E-12	−9·92	−30·80
AB	AEST-36-D-03	−38·26	−19·65
		<i>Δcpgb-1</i>	
		MA	RT
AB	AEST-07-B-12	32·26	−1·16
AB	AEST-11-D-08	−5·21	−12·48
AB	AEST-27-H-09	−8·75	−13·13
AB	AEST-29-E-12	−9·92	−30·80
AB	AEST-36-D-03	−38·26	−19·65
		EP155/CHV1-EP713	
		MA	RT
AV	AEST-09-G-04	−6·68	−17·84
AV	AEST-29-C-07	−6·66	−9·75
AV	AEST-32-A-06	−14·51	−259·27
AV	AEST-38-A-06	−6·66	−15·91
		<i>Δcpgb-1</i>	
		MA	RT
BV	AEST-07-D-09	−8·46	−16·47
BV	AEST-11-F-11	−3·68	−4·65
BV	AEST-17-B-09	5·92	−26·63
BV	AEST-38-C-10	38·78	190·46
		<i>Δcpg-1</i>	
		MA	RT
ABV	AEST-03-G-06	−8·00	−86·52
ABV	AEST-05-B-03	21·77	13·83
ABV	AEST-09-E-04	−17·94	−478·27
ABV	AEST-30-C-09	−11·96	−14·77
ABV	AEST-35-C-02	−23·18	−73·26
		<i>Δcpgb-1</i>	
		MA	RT
ABV	AEST-03-G-06	−8·00	−86·52
ABV	AEST-05-B-03	21·77	13·83
ABV	AEST-09-E-04	−17·94	−478·27
ABV	AEST-30-C-09	−11·96	−14·77
ABV	AEST-35-C-02	−23·18	−73·26
		EP155/CHV1-EP713	
		MA	RT
ABV	AEST-03-G-06	−8·00	−86·52
ABV	AEST-05-B-03	21·77	13·83
ABV	AEST-09-E-04	−17·94	−478·27
ABV	AEST-30-C-09	−11·96	−14·77
ABV	AEST-35-C-02	−23·18	−73·26

relative G-protein subunit accumulation in *C. parasitica* (Parsley *et al.*, 2003). Our results permitted a measurement of the amount of this subunit in the presence of CHV1-EP713, determined to be 25 % of that present in uninfected EP155 mycelium. Additionally, we detected a similar reduction in the relative quantity of CPGB-1 protein. This represents the first indication of hypovirus-mediated alteration of the levels of a G β subunit and is further evidence of a considerable modulation of G-protein-signalling pathways in general by the hypovirus CHV1-EP713. Given the fact that in both cases we observed the transcript levels to be slightly elevated, it is likely that these changes are the result of increased turnover of the proteins in question. Whether this is an effect of the reduction of one subunit, which then results in the corresponding reduction of the putative functional partner (as described by Parsley *et al.*, 2003) or represents hypovirus-mediated attenuation of both G-protein components, is presently unclear.

Combined observations from the three hybridization conditions analysed in this study illuminated a considerable overlap in the expression profile changes that occurred in the absence of CPG-1 or CPGB-1, or in the presence of CHV1-EP713. Of the 100 genes commonly changed between Δ *cpg-1* and Δ *cpgb-1* (class AB), almost half (45 %) were also changed in the presence of hypovirus (class ABV). Of particular note, 43 of these 45 genes were coordinately changed in all three scenarios, suggesting a considerable influence of CHV1-EP713 upon G-protein-signalling. CHV1-EP713 appears to affect CPG-1- and CPGB-1-mediated pathways to an equal extent since classes AV (17 genes) and BV (11 genes) are of similar size, and both are much smaller than ABV. This is consistent with the observation that CHV1-EP713 infection reduces the accumulation of both subunits and reflects a general modulation of G-protein-controlled pathways. Also apparent was the generally larger magnitude of the changes observed in either the Δ *cpg-1* or Δ *cpgb-1* strains compared to CHV1-EP713-infected EP155. This presumably reflects the reduction in abundance of these subunits at the protein level in the presence of hypovirus, with the effects on genes targeted by the G-protein-signalling pathway becoming more pronounced in the complete absence of those proteins.

Previous analysis of the changes wrought by hypovirus infection (Allen *et al.*, 2003) had speculated on the possible involvement of stress response-related proteins, including homologues of heat-shock protein 70 (HSP70) and glutathione S-transferase (GST) on fungal phenotype and virulence. HSP70 (AEST-38-C-04) was downregulated in the presence of CHV1-EP713, while GST (AEST-38-H-09) was upregulated, and both transcripts behave identically in each of the G-protein knockout strains. These results suggest that: (i) either HSP70- and GST-like transcripts may be controlled by G-protein-responsive pathways; or (ii) the deletion of a key regulating component such as a G α or G β subunit results in a stress response that has some similarities to the presence and replication in the

cytoplasm of the 12.7 kb RNA genome of the hypovirus CHV1-EP713.

Allen *et al.* (2003) also noted the specific alteration of homologues of three transcription factors in the presence of CHV1-EP713: Pro1 (regulation of fruiting body development and asexual sporulation in *Sordaria macrospora*; Masloff *et al.*, 2002), HoxX (regulation of hydrogenase biosynthesis in *Bradyrhizobium japonicum*; Durmowicz & Maier, 1997) and Mst12 (regulation of growth of infectious hyphae in *Magnaporthe grisea*; Park *et al.*, 2002). Most interestingly, only Mst12 shows similar regulation in the absence of CPG-1 and CPGB-1. Mst12 was identified based on its similarity to the Ste12 gene from *S. cerevisiae*, a transcription factor that is activated in response to stimulation by mating pheromones through a G-protein-coupled receptor, and leads to the induction of various genes required for mating. In this respect, it is interesting to note that both CHV1-EP713-infected EP155 and the Δ *cpg-1* strain exhibit female infertility (Anagnostakis, 1984; Gao & Nuss, 1996). Thus, the *C. parasitica* Mst12 homologue represents an intriguing candidate that is likely to play a role in modulating the expression of genes important for mating and virulence in response to a G-protein-initiated cascade.

Protease secretion is an important component of pathogenic fungal behaviour (reviewed by Monod & Borg-von, 2002). Specifically, production of secreted aspartyl proteinases was shown to be crucial for virulence, tissue invasion, adhesion and interference with host defence systems by the human pathogen *Candida albicans* (Agabian *et al.*, 1994; Hoegl *et al.*, 1996; Hube *et al.*, 1994; Ruchel *et al.*, 1992). Forty-nine proteases were identified during the analysis of *C. parasitica* EST sequences by Dawe *et al.* (2003) that were used to prepare the spotted cDNA microarray employed in this study. Eleven of these 49 transcripts (22 %) were observed to be changed in abundance in at least one of the tested strains. However, this population of 49 proteases includes 13 predicted acid-proteases of which six (46 %: EapA, -B and -C plus AEST-28-E-10, -29-E-12 and -35-B-04) were changed. Intriguingly, all six are changed in both G-protein-deleted mutants (all are downregulated), but only two are similarly altered in the presence of hypovirus. Previous studies have demonstrated that null mutants of EapA and EapC produced altered extracellular proteolytic profiles, with EapA contributing 97 % of the proteolytic activity detected in *C. parasitica* culture medium (Jara *et al.*, 1996; Razanamparany *et al.*, 1992). However, information concerning the virulence of these strains is presently unavailable. These data provide evidence for modulation of protease production by G-protein-signalling pathways and are consistent with the reported reduction in extracellular protease production by mutants of *Botrytis cinerea* lacking the G α subunit BCG-1 (Gronover *et al.*, 2001). Our information did not permit a comprehensive analysis of potentially secreted proteases since only partial cDNA sequences were available (Dawe *et al.*, 2003). However, we

conclude that downregulation of genes encoding secreted acid proteases may contribute to the reduction in virulence observed in the $\Delta cpg-1$ and $\Delta cpgb-1$ strains of *C. parasitica*. Given the importance of EapA to external proteolytic activity and considering that this transcript is also down-regulated by hypovirus infection, the above conclusion might be valid for CHV1-EP713-infected mycelium as well.

In this study, we have presented data that illustrate the considerable crosstalk and overlap between downstream targets of both G α - and G $\beta\gamma$ -signalling in the filamentous fungus *C. parasitica*. Furthermore, we have shown that global transcriptional changes brought about by the presence of the hypovirus CHV1-EP713 bear a striking similarity to those noted for strains deficient in G-protein-signalling. Coupled with our observation of a reduced accumulation of G-protein components CPG-1 and CPGB-1, these data support the notion that interference of G-protein-mediated signal transduction pathways provides for a major contribution to the phenotype of hypovirus-infected mycelium. Furthermore, our studies have illuminated future directions that will provide for more detailed analysis of pathways that impact fungal virulence and also a better understanding of host-virus interactions at a molecular level.

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