The Role of Glutathione S-Transferase GliG in Gliotoxin Biosynthesis in Aspergillus fumigatus

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SUMMARY

Gliotoxin, a redox-active metabolite, is produced by the opportunistic fungal pathogen Aspergillus fumigatus, and its biosynthesis is directed by the gli gene cluster. Knowledge of the biosynthetic pathway to gliotoxin, which contains a disulfide bridge of unknown origin, is limited, although L-Phe and L-Ser are known biosynthetic precursors. Deletion of gliG from the gli cluster, herein functionally confirmed as a glutathione S-transferase, results in abrogation of gliotoxin biosynthesis and accumulation of 6-benzyl-6-hydroxy-1-methoxy-3-methylene-piperazine-2,5-dione. This putative shunt metabolite from the gliotoxin biosynthetic pathway contains an intriguing hydroxyl group at C-6, consistent with the hypothesis that this group is derived from the gliotoxin biosynthetic pathway via addition of the glutathione thiol group to a reactive acyl imine intermediate. Complementation of gliG restored gliotoxin production and, unlike gliT, gliG was found not to be involved in fungal self-protection against gliotoxin.

INTRODUCTION

The biosynthesis of fungal secondary metabolites frequently involves nonribosomal peptide synthetases and a range of associated enzymes that is often encoded by gene clusters (Maiya et al., 2006; Stack et al., 2007). An example of one such nonribosomal peptide made by fungi is the epipolythiodioxopiperazine (ETP), gliotoxin 1 (Figure 1), which contains a transannular disulfide bridge of unknown origin (Fox and Howlett, 2008). Primarily due to the disulfide bridge, gliotoxin exhibits antimicrobial, immunosuppressive, and angiogenic properties, and its various biological activities have been the subject of significant investigation (Bernardo et al., 2003; Li et al., 2006; Choi et al., 2007). It has also attracted attention as a biomarker of fungal disease and contaminated animal feedstuffs (Lewis et al., 2005; Pena et al., 2010).

The gli biosynthetic cluster, which directs gliotoxin production in Aspergillus fumigatus, was identified in 2004 and is now known to contain 13 genes (Gardiner et al., 2004; Schrettl et al., 2010). Subsequent work showed that the coordinated expression of constituent genes was associated with gliotoxin production, and targeted disruption of the genes encoding a transcriptional regulator (gliZ) and a nonribosomal peptide synthetase (gliP) abolished gliotoxin biosynthesis in A. fumigatus (Gardiner and Howlett, 2005; Cramer et al., 2006; Bok et al., 2006; Kupfahl et al., 2006). Biochemical studies of purified recombinant GliP demonstrate that it is capable of assembling an L-Phe-L-Ser dipeptidyl thioester intermediate tethered to a thiolation domain within the enzyme. This intermediate undergoes slow conversion to the corresponding diketopiperazine 2, which is probably the first free intermediate in gliotoxin biosynthesis (Balibar and Walsh, 2006) (Figure 1). Although the gene encoding the proposed ABC transporter export system for gliotoxin (gliA) has not been disrupted in A. fumigatus, deletion of an ortholog, sirA, in Leptosphaeria maculans surprisingly resulted in increased secretion of sirodesmin, an ETP structurally related to gliotoxin (Gardiner et al., 2005a). The sirA mutant exhibited increased sensitivity to exogenous sirodesmin and gliotoxin, and expression of gliA from A. fumigatus in L. maculans ΔsirA conferred resistance to gliotoxin, but not sirodesmin. More recently, a flavin-dependent oxidoreductase, encoded by gliT within the gliotoxin biosynthetic gene cluster, has been shown to completely protect A. fumigatus against exogenous gliotoxin (Schrettl et al., 2010; Scharf et al., 2010). Gliotoxin production was also abrogated in a gliT mutant of A. fumigatus and transformation of A. nidulans and Saccharomyces cerevisiae, respectively, with gliT conferred gliotoxin resistance on these gliotoxin-sensitive fungal species (Schrettl et al., 2010). Biochemical studies with purified recombinant GliT show that it catalyzes transannular disulfide formation in the dithiol precursor 3, which is the final step in gliotoxin biosynthesis (Schrettl et al., 2010).

The role of other genes within the gliotoxin biosynthetic gene cluster, either in self-protection against, or biosynthesis of, gliotoxin, remains to be fully elucidated (Gardiner et al., 2005b). In particular the origin and mechanism of incorporation of the sulfur atoms into gliotoxin are unclear. Although it has been demonstrated that [3,3-2H2]L-lysine, [3,3-2H2]O-prenyl-L-tyrosine, [3,3,5\(^{-2}H\)\)O-prenyl-L-tyrosine, and [5,5-2H2]phomamide can be incorporated into sirodesmin PL, it has not been possible to conclusively identify—by feeding studies with isotope-labeled precursors—the metabolic origin of the sulfur atoms in this ETP (Pedras and Yang, 2009). The diketopiperazine alkaloid
Figure 1. Proposed Biosynthetic Pathway for Gliotoxin Biosynthesis
Following GliP-mediated conjugation of L-Phe and L-Ser (Balibar and Walsh, 2006), a series of hydroxylations and dehydration reactions result in reactive acyl imine intermediate formation. Thiolation (GliG-mediated using GSH), possibly followed by sequential GluI peptidase and GliM thioesterase activity, then occurs to yield the sulfurized intermediate. Subsequent tailoring reactions lead to gliotoxin formation. Compound 4 is isolated as an off-pathway metabolite as a result of intermediate instability following gliG deletion. See also Figures S2–S9.

The role of gliG (AFUA_6G09690; http://www.cadre-genomes.org.uk/) within the gli cluster, which in silico analysis predicts encodes a glutathione S-transferase (GST), is unclear. It could be involved in biosynthetic thiolation of an acyl imine intermediate or auto-protection against gliotoxin (Figure 1). Normally, GSTs catalyze conjugation of glutathione (GSH) to toxic (chemically reactive) metabolites to facilitate detoxification and cellular secretion. Although GSTs have received significant attention in mammalian and plant systems (Hayes et al., 2005; Dixon et al., 2010), their presence and function in fungi have only recently been studied (Burns et al., 2005; Morel et al., 2009). The low- percentage sequence identity of GliG to other putative GSTs encoded within the A. fumigatus genome (Cramer et al., 2006) (approximately 30%) may indicate a function for GliG that is distinct from the other GSTs (Nierman et al., 2005). To investigate the function of gliG in A. fumigatus, it was disrupted, and the phenotype of the mutant with respect to gliotoxin biosynthesis and auto-protection was examined.

RESULTS
Deletion and Complementation of gliG in A. fumigatus
Homologous transformation using the bipartite marker technique (Nielsen et al., 2006), with modifications, was used to generate ΔgliG mutants in both A. fumigatus ΔakuB (da Silva Ferreira et al., 2006) and AF293 (Nierman et al., 2005) backgrounds. Here, the A. fumigatus strains were cotransformed with two DNA constructs, each containing an incomplete fragment of the pyrithiamine (ptrA) resistance gene (Kubodera et al., 2000) fused to 1.2 kb (5) and 1.0 kb (3) of gliG-flanking sequences (Figure 2). These constructs were generated by PCR and characterized by DNA sequence analysis that confirmed the replacement of gliG by overlapping ptrA regions on intact 5’ and 3’-flanking regions, respectively, Southern analysis was used to screen 37 putative transformants for gliG (negative) and ptrA (positive) colonies with a 2124 bp XbaI restriction fragment in place of the wild-type 1668 bp XbaI restriction fragment (Figure 2). This led to identification of a gliG mutants (A. fumigatus ΔgliGΔakuB and ΔgliGΔAF293). Complementation of the A. fumigatus ΔgliGΔAF293 mutant was achieved by transformation with a vector containing intact gliG and the phleomycin resistance gene using selection on phleomycin-containing media. Southern analysis of the complemented strains produced the expected EcoRV restriction fragments of 3.4 and 1.5 kb, respectively (Figure 2).
These complemented strains (gliGC), termed 15.1, 15.4, and 17.1, exhibited wild-type features in all subsequent experiments. Northern analysis confirmed that gliG transcripts are absent from DgliGakuB and DgliGAF293 but present in A. fumigatus wild-type and gliGC (Figure 3).

Phenotypic Analysis

Exposure of A. fumigatus wild-type and ΔgliG conidia to exogenous gliotoxin (10–50 μg/ml) showed that both strains grew equally well (Figure 4), thereby confirming that gliG does not play a role in self-resistance to gliotoxin. Observation of A. fumigatus wild-type and ΔgliG conidia in the presence of H2O2 (1, 2, and 5 mM), voriconazole (0.15 and 0.25 μg/ml), and amphotericin B (1, 2, and 5 μg/ml) showed that both wild-type and mutant grew at identical rates (data not shown), thus excluding gliG involvement in protection against oxidative stress and antifungal agents. Exposure to conidia of A. fumigatus ΔgliG induced greater melanization in the Galleria mellonella virulence model (Reeves et al., 2004) compared to wild-type or gliGC (15.1, 15.4, and 17.1), although no statistical significance was observed in overall virulence among wild-type, mutant, or complemented strains (data not shown).

A. fumigatus ΔgliG Does Not Produce Gliotoxin

Organic extracts from 48-hr cultures of A. fumigatus wild-type, ΔgliGakuB, ΔgliGAF293, and gliGC were prepared and analyzed by LC-ToF MS (Figure 5) and comparative RP-HPLC analysis (see Figure S1 available online). It is clear from Figure 5 that although gliotoxin was produced (mean ± SD: 784.9 ± 869.88 μg/ml) by A. fumigatus wild-type (retention time [RT]= 14.8 min, Figure S1), no gliotoxin was detectable in organic extracts from the A. fumigatus ΔgliG strains. However, an alternative metabolite, termed M12.3, was evident in ΔgliG organic
extracts, with Rf = 12.3 min (Figure 6). LC-ToF MS analysis also confirmed the restoration of gliotoxin production in gliGΔgliG2 mutant by northern analysis. Wild-type (lane 1) and ΔgliG (lane 2) cultures were grown for 21 hr, followed by gliotoxin addition (3 hr). Wild-type (lane 3) ATCC6645 cultures were grown for 48 hr in AMM. Northern analysis was confirmed, and absence of gliG expression in ΔgliG2ΔgliG is evident. Equal RNA loading/track was shown by rRNA subunit presence.

(A) Absence of gliG expression was confirmed in A. fumigatus ΔgliG2ΔgliG2 by northern analysis. Wild-type (lane 1) and ΔgliG2ΔgliG2 (lane 2) cultures were grown for 21 hr, followed by gliotoxin addition (3 hr). Wild-type (lane 3) ATCC6645 cultures were grown for 48 hr in AMM. Northern analysis was confirmed, and absence of gliG expression in ΔgliG2ΔgliG2 is evident. Equal RNA loading/track was shown by rRNA subunit presence.

(B) Northern blot analysis of gliG expression in A. fumigatus AF293, ΔgliG2AF293, and gliG2. Lane 1 corresponds to A. fumigatus RNA extracts from 48-hr AMM for AF293 wild-type. Lane 2 shows RNA extracts from 48-hr AMM for A. fumigatus ΔgliG2AF293. Lanes 3–5 correspond to A. fumigatus RNA extracts from 48-hr AMM for three independent ΔgliG2-complemented strains. Equal RNA loading/track was shown by rRNA subunit presence.

A Gliotoxin Biosynthetic Shunt Metabolite Is Secreted by A. fumigatus ΔgliG

LC-ToF MS analyses of organic extracts of ΔgliG2AF293 indicated the presence of a compound (M12.3) with m/z = 263.1 that was absent from wild-type A. fumigatus and the complemented gliG mutant (Figure 6). Subsequent high-resolution LC-ToF MS analysis of M12.3, purified by preparative TLC (Figures 6A–6C), gave the molecular formula C13H14N2O4 for this compound (m/z calculated for C13H14N2O4+: 263.1026; m/z found: 263.1027) (Figure 6D). The compound is proposed to be 6-benzyl-6-hydroxy-1-methoxy-3-methylenepipеразине-2,5-dione 4 (Figure 1), on the basis of NMR spectroscopic analysis.

Analysis of 1H and 13C, DEPT, COSY, HMBC, and HMQC spectra revealed that the compound contains two amide carbonyl groups (Δδ5.1175.3, CONH; 161.1, CONOCH3), a benzyl group (Δδ5.46.5, CH2Ph), a hydroxyl group at position 6 (Δδ5.83.5, NHOC(OH)Bn; Δδ5.4, 4.93, NHOC(OH)Bn), a methoxy group (Δδ62.2, OCH3; Δδ3.64, OCH3), and a 1,1-disubstituted alkenone (Δδ100.1, C=CH2; 134.2, C=CH2; Δδ5.50.0, 5.33, C=CH2) (Figures 1 and Table 1; Figure S2–S9). The proton of the hydroxyl group, at position 6, could be observed in the 1H-NMR spectrum measured in CD3CN, (Δδ14.93 [NHOC(OH)Bn]). This signal disappeared upon shaking with D2O (Figure S3). We observed a ΔδC value of 83.5 for C-6 of the compound that compares very favorably with reported ΔδC values of 82.9 and 87.2 for the almost identical C-6 carbons in similar compounds (Buysens et al., 1996; King et al., 2003). In the 1H-NMR spectrum in CD3CN, the CONH proton signal at Δδ7.50.2 overlaps with the signals due to the aromatic protons, but in CDCl3 the CONH signal is shifted away from the aromatic signals to Δδ7.50. This shift in the CONH signal allowed us to distinguish between structure 4 and an alternative structure with the methoxy group attached to N-4 rather than N-1. The COSY spectrum in CDCl3 showed a clear coupling between the CONH signal at Δδ7.50.2 and the methylene protons (C = CH2) (Figure 1; Figure S6). This long-range coupling, over four bonds, indicated that the NH group was located at position 4, adjacent to the disubstituted alkene (location of the NH group at position 1 would require a five bond coupling to the methylene protons). In addition the HMBC correlation data obtained in CDCl3 showed a coupling between the CONH proton signal at Δδ7.50.2 and C-5/C-3 (Figure 1 and Table 1), which is consistent with structure 4. The alternative structure with the methoxy group at position 4 would be expected to show couplings between the CONH signal and C-2 and C-6 in the HMBC spectrum, which was not observed (Figure S9). Finally, no deep-violet color was observed when a ferric chloride test for the presence of hydroxamic acids was performed (Shin et al., 1975), confirming the absence of a hydroxamic acid group in the compound.

Feeding experiments with [3-13C]-L-Phe and subsequent LC-ToF MS analysis strongly indicated that L-Phe is incorporated into 4 (Figure 6E). A substantial enhancement of the Ch2Ph signal in the 13C-NMR spectrum of the labeled material (Figures S4B and S5B) confirmed L-Phe as a precursor of compound 4. No uptake of compound 4 was detectable by RP-HPLC analysis in either A. fumigatus AF293 mycelia or protoplasts (data not shown). This precluded M12.3 incorporation experiments in A. fumigatus wild-type.

Recombinant GliG Exhibits GST and Reductase Activity

Protein expression plasmid pPXaglG consisting of the vector pProEx-HtB containing the coding sequence for gliG was used to transform Escherichia coli DH5α, and expression was induced by the addition of 0.6 mM IPTG. rGliG was present in cell lysate pellet, indicating insolubility, and was purified using differential extraction, with a yield of approximately 12 mg/g of E. coli. SDS-PAGE analysis confirmed a molecular mass of 25 kDa for
rGlIG (Figure S10). The identity of purified rGlIG was confirmed by peptide mass fingerprinting. Several peptides corresponding to the theoretical protein sequence (26.5% sequence coverage; data not shown) were identified. Following large-scale overproduction, rGlIG was solubilized and subjected to serial dialysis at 250 μg/ml, yielding 67% rGlIG recovery (170 μg/ml). Post-dialysis, rGlIG exhibited GST activity with 1,2-epoxy-3-(4-nitrophenoxy)-propane (EPNP Specific Activity [S.A.] [mean ± SD] = 2.3 ± 0.122 U/mg), 1-chloro-2,4-dinitrobenzene (CDNB S.A. = 0.20 ± 0.001 U/mg), and 3,4-dichloro-nitrobenzene (DCNB S.A. = 0.09 ± 0.001 U/mg). rGlIG also exhibited glutathione reductase activity (S.A. = 0.01 ± 0.002 U/mg). Whole-protein lysates from A. fumigatus DgliG exhibited 17% less activity toward EPNP than those from A. fumigatus wild-type (S.A. = 0.12 ± 0.02 U/mg versus 0.145 ± 0.011 U/mg, respectively).

**DISCUSSION**

The biosynthetic pathway to gliotoxin has, before now, been unclear. Using a gene deletion strategy, our work has demonstrated that GlIG, which exhibits GST activity and is encoded by a gene within the gli cluster, is essential for gliotoxin production in A. fumigatus. Furthermore, the absence of GlIG results in the accumulation and secretion of the shunt metabolite 6-benzyl-6-hydroxy-3-hydroxymethylpiperazine-2,5-dione 4, which lacks both of the sulfur atoms incorporated into the disulfide bridge of gliotoxin. Genetic complementation of the A. fumigatus gliG mutant restored gliotoxin production. Unlike some of the other genes in the gli cluster studied to date (e.g., gliT or gliA), gliG is not involved in the auto-protection of A. fumigatus against exogenous gliotoxin. Taken together, our data suggest that GlIG is involved in the introduction of sulfur into 6-benzyl-6-hydroxy-3-hydroxymethylpiperazine-2,5-dione 5 (Figure 1), the likely precursor of the shunt metabolite 4.

The gliG gene was deleted from both A. fumigatus AF293 and A. fumigatus ΔakuB in order to elucidate its function in gliotoxin metabolism, a strategy noted by others for the functional dissection of the gliotoxin biosynthetic pathway (Patron et al., 2007; Fox and Howlett, 2008). Expression of gliG, along with other genes in the gli cluster, has been shown to be coincident with gliotoxin formation (Gardiner and Howlett, 2005). Moreover, Cramer et al. (2006) demonstrated that low-level gliG expression was evident in the A. fumigatus ΔgliP termed ARC2 that, along with other gli cluster components (except gliP), was upregulated upon exposure to gliotoxin (20 μg/ml) for 24 hr. This has led to the suggestion that gliotoxin plays a role in the regulation of its own biosynthesis. Cramer et al. (2006) also observed entire gli cluster expression in A. fumigatus wild-type (AF293) in the presence of gliotoxin (20 μg/ml). In the present work, northern analysis confirmed the presence and absence of gliG expression in A. fumigatus AF293 and ΔgliG AF293, respectively, in the absence

![Figure 4. Effect of Gliotoxin on the Growth of A. fumigatus AF293, ΔgliG, and ΔgliT, Respectively](image-url)

Radial growth (mean ± standard deviation) was measured up to 72 hr (n = 3). No statistically significant difference was observed on the effect of gliotoxin (0, 10, 30, and 50 μg/ml) (A–D) on A. fumigatus ΔgliG growth relative to wild-type.
of added gliotoxin. However, gliotoxin addition was necessary to detect gliG expression in *A. fumigatus ΔakuB* wild-type, although no transcript was detectable in *A. fumigatus ΔgliGΔakuB* by northern analysis. Coincident with absence of gliG expression, both *A. fumigatus ΔgliG* strains generated in this work were unable to produce gliotoxin, although a gliotoxin-related shunt metabolite (M12.3) 4 was identified in both deletion strains. Disruption of gliP and gliZ, respectively, also abrogates gliotoxin production. However, no gliotoxin-related metabolites were detected in these studies (Cramer et al., 2006; Kupfahl et al., 2006). Thus, our results extend the number of genes in the gli cluster confirmed to play a role in gliotoxin biosynthesis. Along with gliG expression, gliotoxin production was restored in *A. fumigatus ΔgliGΔAF293* upon introduction of the entire gliG coding sequence. Interestingly, no gliG-complemented strains were recoverable from *A. fumigatus ΔgliG* disrupted in the akuB background. Bok et al. (2006) also observed restoration of gliotoxin production upon complementation of an
A. fumigatus gliZ mutant with the gliZ coding sequence. Moreover, complementation of a gliP mutant of A. fumigatus with gliP also restored gliotoxin biosynthesis and secretion (Cramer et al., 2006; Kupfahl et al., 2006).

Secreted metabolites may be involved in maintenance of competitive advantage against other fungi, and it has been speculated that toxin-resistance genes may exist in fungi to protect against the autotoxicity of such metabolites (Losada et al., 2009). Given the role of GSTs in reactive metabolite detoxification in animals, plants, and fungi (Hayes et al., 2005; Dixon et al., 2010; Burns et al., 2005), it was conceivable that gliG is involved in self-protection against gliotoxin, as has previously been demonstrated by expression of A. fumigatus gliA in L. maculans (Gardiner et al., 2005a). However, A. fumigatus ΔgliGAF293 did not exhibit increased sensitivity to gliotoxin compared to A. fumigatus wild-type. Thus, gliG, and most likely all orthologs found in other ascomycetes (Patron et al., 2007; Fox and Howlett, 2008), does not appear to be primarily involved in self-protection against gliotoxin. This observation prompted us to investigate a role for gliG in gliotoxin biosynthesis.

Although Balibar and Walsh (2006) have shown that GliP can assemble 6-benzyl-3-hydroxymethylpiperazine-2,5-dione 2 (Figure 1) from L-Phe and L-Ser, it is unclear whether this diketopiperazine is an intermediate in gliotoxin biosynthesis. Indeed, to our knowledge, no free reaction intermediates in gliotoxin biosynthesis have been detected to date, as previously noted by Fox and Howlett (2008). Herein we report that 6-benzyl-6-hydroxy-1-methoxy-3-methylenepiperazine-2,5-dione 4 accumulates in and is secreted by a gliG mutant of A. fumigatus. Based on the structural similarity of 4 to 1 and 2, it seems likely that 4 is a shunt metabolite from the gliotoxin biosynthetic pathway. We hypothesize that 6-benzyl-3-hydroxymethylpiperazine-2,5-dione 2 undergoes hydroxylation at C-6, catalyzed by the cytochrome P450s GliC or GliF to yield 5 (Figure 1). Hydroxylation of 5 at N-1, catalyzed either by GliC/GliF or more likely a nonspecific monooxygenase not encoded by a gene within the gliotoxin biosynthetic gene cluster, followed by O-methylation, perhaps catalyzed by GliM, and elimination of water from C-3/C-7 would give the shunt metabolite 4 (Figure 1). It is possible that N1 functionalization could be off...
Gliotoxin Biosynthesis in *Aspergillus fumigatus*

### Table 1. NMR Data for M12.3 (6-benzyl-6-hydroxy-1-methoxy-3-methylenepiperezine-2,5-dione-4) in Cδ3CN Unless Indicated

<table>
<thead>
<tr>
<th>Position</th>
<th>¹H (mult, J in Hz)</th>
<th>¹³C (mult)</th>
<th>HMBC (H to C)</th>
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<tr>
<td>1(N)-OCH₃</td>
<td>3.63 (s)</td>
<td>62.2 (q)</td>
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<tr>
<td>2</td>
<td></td>
<td>157.2 (s)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>134.2 (s)</td>
<td></td>
</tr>
<tr>
<td>4-NH</td>
<td>7.23</td>
<td></td>
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<tr>
<td>4-NH₂</td>
<td>7.50</td>
<td>C-5, 3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>161.1 (s)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>83.5 (s)</td>
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<tr>
<td>6-OH</td>
<td>4.93 (s)</td>
<td>C-6, 8</td>
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<tr>
<td>7</td>
<td>5.00 (s), 5.33 (s)</td>
<td>100.1 (t)</td>
<td>C-2, 3</td>
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<tr>
<td>8</td>
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<td>46.5 (t)</td>
<td>C-5, 6, 9, 10, 14</td>
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<tr>
<td>10, 14</td>
<td>7.17–7.29 (m)³</td>
<td>131.4 (d)³</td>
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<td>11, 13</td>
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<td>7.17–7.29 (m)³</td>
<td>128.2 (d)³</td>
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*¹ Overlapping the signals of the phenyl group.
*² In CDCl₃.
*³ Overlapping the NH signal.
* Assignments of carbons are interchangeable.

Most studies pertaining to gliotoxin have focused on its role in mediating fungal virulence and the mechanisms of action by which this occurs. Although the roles of GliP and GliT in the first and last steps of gliotoxin biosynthesis, respectively, have been investigated in vitro and in vivo, to our knowledge, no further information on gliotoxin biosynthesis has been forthcoming. Here, we demonstrate that disruption of *gliG* within the gliotoxin biosynthetic gene cluster of *A. fumigatus* results in the secretion of a shunt metabolite, 6-benzyl-6-hydroxy-1-methoxy-3-methylenepiperazine-2,5-dione. We show that GliG exhibits glutathione S-transferase activity, and the presence of a hydroxyl group at C-6 of the shunt metabolite leads us to propose that elimination of water from N-1/C-6 of a C-6 hydroxylated piperazine-2,5-dione intermediate yields an acyl imine, which undergoes GliG-mediated addition of glutathione to acyl imine-containing intermediates is proposed to be a key step in gliotoxin biosynthesis. To our knowledge, this is the first time that such a reaction has been suggested to play a pivotal function in ETP biosynthesis.

**SIGNIFICANCE**

GSTs form homodimers (Sheehan et al., 2001). Nonreducing SDS-PAGE analysis of rGliG identified homodimeric and monomeric forms, indicating that rGliG is, like other GSTs, homodimeric. Activity of rGliG toward EPNP, CDNB, and DCNB was observed, verifying the predicted GST activity of this enzyme. GliG exhibited higher activity toward both CDNB and DCNB than GstA, GstB, and GstC, previously cloned and expressed from *A. fumigatus* (Burns et al., 2005). In fact, GstA, GstB, and GstC did not have detectable activity toward DCNB. Interestingly, a low but detectable glutathione reductase activity was observed for rGliG. Although rGliG did not exhibit glutathione peroxidase activity, this was detectable for positive control enzyme, *A. fumigatus* GstB (Burns et al., 2005). Thus, it appears that rGliG exhibits a different pattern of reactivity to other *A. fumigatus* GSTs.

In conclusion we show that the GST, GliG, is involved in the biosynthesis of gliotoxin and not in self-resistance to this ETP. GliG-mediated addition of glutathione to acyl imine-containing intermediates is proposed to be a key step in gliotoxin biosynthesis. The cysteine amino groups allows their condensation with gliotoxin-bound PLP. In conclusion, we propose that GliG mediates the “deprotection” of the cysteine amino groups allows their condensation with gliotoxin-bound PLP. Thus, we propose that GliG catalyzes two pyridoxal-mediated α, β-elimination reactions of 9 to afford 6-benzyl-3-hydroxymethylpiperazine-2,5-dione-3, 6-dithiol 10 (Figure 1). GliN-catalyzed N-4 methylation of 10, followed by epoxidation of the phenyl group, catalyzed by the putative cytochrome P450s GliC or GliF, and epoxide opening with N-1, would yield reduced gliotoxin 3, which has been reported to undergo GliT, FAD, and O₂-mediated oxidation to form gliotoxin 1 (Figure 1) (Wang et al., 2009; Schrettl et al., 2010; Scharf et al., 2010). It is conceivable that the GliJ- and GliI-catalyzed reactions to expose the thiols in 3 may occur after N-4 methylation and epoxidation/epoxide opening, given the potential deleterious effects of intracellular gliotoxin, due to the presence of reactive thiols or a disulfide bridge capable of redox cycling (Waring et al., 1995; Bernardo et al., 2003; Gardiner et al., 2005b; Nishida et al., 2005).


EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and General DNA Manipulation

In general, A. fumigatus strains (Table S1) were grown at 37°C in Aspergillus minimal media (AMM). AMM contained 1% (w/v) glucose as carbon-source, 20 mM L-glutamine as nitrogen-source, and trace elements according to Pontecorvo et al. (1953).

Gene Deletion and Complementation in A. fumigatus

For generating aglG strains, the bipartite marker technique was used (Nielsen et al., 2006). Primer details are provided in Table S2. Briefly, A. fumigatus strains AF293 and akuB were cotransformed with two DNA constructs, each containing an incomplete fragment of a pyrithiamine resistance gene (ptrA) (Tilburn et al., 2005; Kubodera et al., 2000) fused to 1.0 and 1.2 kb of gligG-flanking sequences, respectively. Further details and the gligG complementation strategy are given in Supplemental Experimental Procedures.

Northern Analysis

RNA was isolated using TRI-Reagent (Sigma-Aldrich). Equal concentrations of total RNA (10 μg) were size separated on 1.2% (w/v) agarose-2.2 M formaldehyde gels and blotted onto Hybond N+ membranes (Amersham Biosciences). The hybridization probes used in this study were generated by PCR using primers gligG-7 and gligG-8.

Phenotypic Analysis of A. fumigatus AF293 Wild-Type and aglG Strains

Relative sensitivities of A. fumigatus wild-type, aglG, and gligG were assessed against gliotoxin (10–50 μg/ml) (Schrettl et al., 2010; Losada et al., 2009), voriconazole (0.15–0.25 μg/ml), H2O2 (1, 2, and 5 mM), and amphotericin B (1, 2, and 5 μg/ml). See Supplemental Experimental Procedures for further details.

Virulence Model

G. mellonella larvae (n = 10) were inoculated into the hind pro-leg with 105 A. fumigatus conidia in 20 μl (per larva) (Reeves et al., 2004). Mortality rates were recorded for 72 hr postinjection. See Supplemental Experimental Procedures for further details.

Analysis of Gliotoxin Production

To analyze gliotoxin production, A. fumigatus wild-type, ΔglgI and complemented strains were grown at 37°C for 72 hr in AMM. Supernatants were chloroform extracted, and fractions were evaporated to complete dryness, followed by resolubilization in methanol and analysis by RP-HPLC or LC-MS, as described by Reeves et al. (2004) and Schrettl et al. (2010).

LC-MS Analysis

Electrospray MS analysis of gliotoxin was performed as described previously (Schrettl et al., 2010). LC-ToF MS was performed on an Agilent HPLC 1200 series and injected (injection volume: 10 μl) using electrospray ionization inputted into a time-of-flight chamber (Agilent). The LC separation was done on a DB C18 column (4.6 x 150 mm) using a water/acetonitrile (both containing 0.1% [v/v] formic acid) gradient at a flow rate of 0.5 ml/min. The gradient was started at 50% (v/v) acetonitrile, which was increased to 100% acetonitrile in 10 min; 100% acetonitrile was maintained for 5 min before the gradient was returned to starting conditions. Spectra were collected at 0.99 spectra per second.

Extraction and Purification of the Gliotoxin Shunt Metabolite

A. fumigatus ΔglgI cultures were grown at 37°C for 48 hr in AMM. Culture supernatants were extracted twice with chloroform. Organic extracts were evaporated to dryness and resolubilized in methanol. A layer of the methanolic supernatants were extracted twice with chloroform. Organic extracts were evaporated to dryness and evaporated to dryness and resolubilized in either CD3CN or CDCl3 for NMR analysis.

Incorporation of [3-13C]-Phe into M12.3 (Compound 4)

[3-13C]-Phe (10 mg) was added to 24-hr cultures of A. fumigatus ΔglgI (10 ml). The culture supernatants were harvested after a further 24 hr of incubation, and M12.3 was purified as described above.

NMR Spectroscopic Analysis

1H, 13C, DEPT, COSY, HSQC, and HMBC-NMR spectra were recorded in CD3CN or CDCl3, on a Bruker Avance AV300 spectrometer operating at 300 MHz for the 1H nucleus and 75 MHz for the 13C nucleus. 1H and 13C-NMR spectra were also obtained at 500 and 125 MHz, respectively, on a Bruker Avance III 500 MHz spectrometer at 25°C. Chemical shifts are reported in ppm referenced to residual protonated solvent.

Reductive Alkylation of Gliotoxin

An alkylation strategy to specifically label free thiols was used to determine the presence of sulphydryl residues, either prior to or post-reduction with NaBH4, in gliotoxin or related metabolites. Briefly, organic extracts of culture supernatants from A. fumigatus AF293, ΔglgIAF293, and gligG (100 μl), respectively, were treated for 1 hr with NaBH4 (50 mM final) to reduce disulfide bridges prior to alkylation using 5′-iodoacetamidofluorescein (400 nmol) to yield diacetylamido-fluorescein-gliotoxin (GT-(AF)2), which was identified by RP-HPLC analysis (Schrettl et al., 2010).

GlgiG Expression and Activity Analysis

The gliG coding sequence was amplified from A. fumigatus cDNA using primers gliG-F and gliG-R, respectively (Table S2), and cloned into pProEx-Httb vector (Invitrogen). Expression in, and purification from, E. coli was undertaken as previously described for A. fumigatus GsA-C (Burns et al., 2005). GST, and related enzyme activities were determined as previously described (Burns et al., 2005; Habdous et al., 2002; Hoque et al., 2007). Methodology used for protein isolation from A. fumigatus (Carberry et al., 2006; Bradford, 1976) is given in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, ten figures, and two tables and can be found with this article online at doi:10.1016/j.chembiol.2010.12.022.

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