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Genomewide Screening for Genes Associated with Gliotoxin Resistance and Sensitivity in *Saccharomyces cerevisiae*▽

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Gliotoxin (GT) is a secondary fungal metabolite with pleiotropic immunosuppressive properties that have been implicated in *Aspergillus* virulence. However, the mechanisms of GT cytotoxicity and its molecular targets in eukaryotic cells have not been fully characterized. We screened a haploid library of *Saccharomyces cerevisiae* single-gene deletion mutants (4,787 strains in EUROSCARF) to identify nonessential genes associated with GT increased resistance (GT-IR) and increased sensitivity (GT-IS). The susceptibility of the wild-type parental strain BY4741 to GT was initially assessed by broth microdilution methods using different media. GT-IR and GT-IS were defined as a fourfold increase and decrease, respectively, in MIC, and this was additionally confirmed by susceptibility testing on agar yeast extract-peptone-glucose plates. The specificity of GT-IR and GT-IS mutants exhibiting normal growth compared with the wild-type strain was further tested in studies of their susceptibility to conventional antifungal agents, cycloheximide, and H_2O_2. GT-IR was associated with the disruption of genes acting in general metabolism (*OPI1*, *SNF1*, *IFA38*), mitochondrial function (*RTG2*), DNA damage repair (*RAD18*), and vesicular transport (*APL2*) and genes of unknown function (*YGL235W*, *YOR345C*, *YLR456W*, *YGL072C*). The disruption of three genes encoding transsulfuration (*CYS3*), mitochondrial function (*MEF2*), and an unknown function (*YKL037W*) led to GT-IS. Specificity for GT-IR and GT-IS was observed in all mutants. Importantly, the majority (69%) of genes implicated in GT-IR (6/10) and GT-IS (2/3) have human homologs. We identified novel *Saccharomyces* genes specifically implicated in GT-IR or GT-IS. Because most of these genes are evolutionarily conserved, further characterization of their function could improve our understanding of GT cytotoxicity mechanisms in humans.

Invasive aspergillosis (IA) has emerged as a major problem in modern mycology over the past 2 decades (10, 12). Mortality rates associated with IA remain high in patients with leukemia and in transplant recipients (10). *Aspergillus fumigatus* causes the majority of cases of IA. Despite substantial strides in fungal genetics over the past decade, much remains to be learned about the biology and molecular mechanisms of *Aspergillus* virulence in an effort to develop targeted therapeutic strategies.

Gliotoxin (GT), a secondary fungal metabolite with pleiotropic immunosuppressive properties that belongs to the class of epipolythiodioxopiperazines, has been recently implicated in *Aspergillus* virulence (7). The toxicity of GT and other mycotoxins in this class is attributed to the presence of a disulfide bridge, which can (i) inactivate proteins via reaction with thiol groups and (ii) generate reactive oxygen species by redox cycling (7). GT has a wide range of effects on metazoan cells in vitro, largely mediated by induction of apoptosis through (i) inhibition of the NF-κB pathway and (ii) direct activation of the mitochondrial proapoptotic protein Bak, by suppression of superoxide production by phagocytic cells through inhibition of NADPH oxidase activity, and by inactivation of enzymes such as alcohol dehydrogenase, creatine kinase, and farnesyltransferase (7, 19, 23). These activities of GT, coupled with the fact that GT has been detected in the sera of animals with IA and in immunocompromised patients with IA (13, 14), have led to the concept that GT contributes to pathogenesis during invasive *Aspergillus* growth.

Nonetheless, animal studies using different *A. fumigatus* strains that have a deletion in the GT-encoding gene (*Glip*Δ) provided conflicting results on the role of GT in *Aspergillus* pathogenicity. To that end, recent studies demonstrated that *Glip*Δ mutants displayed virulence comparable to that of the parental strain in neutropenic mice immunosuppressed with both cyclophosphamide and corticosteroids (4, 11). In contrast, other investigators have reported a significant reduction in the virulence of *Glip*Δ mutants compared to that of the parental isogenic *A. fumigatus* strains in nonneutropenic corticosteroid-immunosuppressed mice (22, 24). Overall, the aforementioned studies link the virulence properties of GT to its immunosuppressive activity on phagocytic cells. However, the molecular targets of GT action in eukaryotic cells have not been fully characterized.

Saccharomyces cerevisiae is a model eukaryotic fungal organism for which molecular genetics have been well developed. In this study, we performed a high-throughput screening in an *S. cerevisiae* library of single-gene-deletion haploid mutants in order to identify genes associated with GT increased resistance (GT-IR) and GT increased sensitivity (GT-IS). (This paper was presented in part at the 46th Interscience Conference on

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MATERIALS AND METHODS

***S. cerevisiae* deletion strain library.** The *S. cerevisiae* deletion strain library, constructed in the wild-type (wt) parental strain BY4741 haploid background, is from EUROSCARF (Frankfurt, Germany) and has been used previously in similar genomic studies on the molecular mechanisms of action of other compounds and toxins in eukaryotic cells (2, 9, 16, 21). The library is composed of 4,787 strains, each of which carries a defined deletion of a characterized or a putative nonessential open reading frame replaced with the *kanMX4* marker. Only nonessential genes (~82% of the total) are represented in this collection. Strains were stored in 96-well plates at −80°C in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose) supplemented with 15% glycerol.

Screening for deletion mutants with increased GT susceptibilities. GT (Sigma Chemical Co., St. Louis, MO) was dissolved in 100% methanol, and a stock solution (1 mg/ml) was stored at −70°C until used. The MIC of GT for the wt parental strain BY4741 was initially determined by susceptibility testing with YPD medium and a yeast nitrogen base medium (Difco, Detroit, MI) in microtitration plates (Corning, New York, NY) containing serial twofold dilutions of GT (range of GT concentration, 0.03 to 8 μg/ml). The MIC of GT for the wt strain was determined by using two different inocula (low inoculum, final concentration of $\sim 10^3$ yeast cells/well; high inoculum, final concentration of $\sim 10^5$ yeast cells/well).

The primary screen consisted of the analysis of each deletion strain at 0.25× the MIC, the MIC, and 4× the MIC of GT for the wt. Deletion strains were inoculated from frozen stocks into 96-well plates containing 200 μl of YPD medium and were incubated for 2 days at 30°C until the growth was confluent. Strains were diluted in YPD medium in 96-well plates to yield $\sim 10^5$ cells/well. Plates were incubated at 30°C and scored for growth at 24 and 48 h. A positive result was scored as GT-IS if no growth or very weak residual growth was detected at a concentration of 0.25× the MIC for the wt after 48 h. A positive result was scored as GT-IR if confluent growth was detected at 4× the MIC for the wt after 48 h. The functions of the disrupted genes from the GT-IS and GT-IR strains were derived from the Stanford website (http://genome-www4.stanford.edu/cgi-bin/SGD/seqTools).

Secondary screens. The GT-IS and GT-IR strains identified in the initial screen were grown for 2 days at 30°C until growth was confluent. Cultures were adjusted to equal optical densities at 600 nm and inoculated into 96-well plates, and the strains were retested for their susceptibilities to a range of GT concentrations (0.03 to 16 μg/ml) in YPD medium by broth microdilution methods using two different inocula ($\sim 10^5$ and $\sim 10^3$ cells/well). Furthermore, susceptibility studies of GT-IR and GT-IS strains were performed with agar YPD plates supplemented with different concentrations of GT (range, 0.03 to 16 μg/ml) by spotting 5-μl aliquots of a standardized solution of each strain ($\sim 10^5$ yeast cells/ml) and examining the growth following 48 h of growth at 30°C.

In addition, the growth rates of the GT-IS and GT-IR strains were determined at an optical density at 600 nm in triplicate following 24 h of growth, during which time the growth rates were logarithmic, under the same conditions described above. Strains exhibiting defective growth (defined as a growth rate of ≤80% of that of the wt parental strain) or those strains with no significant change in GT susceptibility on retesting (less-than-fourfold decrease or increase in MIC compared to that of the wt strain) were eliminated from further analysis.

Furthermore, the specificities of GT-IS and GT-IR isolates identified in the primary screen were assessed by broth microdilution susceptibility testing with conventional antifungal agents, including amphotericin B deoxycholate (AMB; 0.06 to 2 μg/ml) (commercial compound from an institutional pharmacy), caspofungin (CAS; 0.003 to 8 μg/ml) (commercial compound from an institutional pharmacy), and fluconazole (FLC; 4 to 256 μg/ml) (Pfizer Inc., New York, NY), and the protein synthesis inhibitor cycloheximide (0.025 to 0.2 μg/ml; Sigma Chemical Co.) in YPD medium.

We also tested the susceptibilities of these strains relative to that of the wt strain against the oxidative agent H_2O_2 (institutional pharmacy). Susceptibility to H_2O_2 was assessed by disk diffusion testing in YPD plates containing 10 μl of 30% H_2O_2 solution. Two-hundred-microliter volumes of a standardized suspension of yeast cells (10^6 cells/ml) of each mutant and the wt strain isolate were plated. After the plates were allowed to dry, a sterile 0.25-inch paper disk (Schleicher and Schuell, Keene, NH) was placed on the agar surface and inoculated with 10 μl of 30% H_2O_2. The plates were incubated at 30°C, and the radii of the zones of inhibition were measured at 48 h by using a micrometer. AMB (final concentration, 10 μg/ml) was used as a control. Three independent experiments were performed at different time. The MICs of GT and each of the other compounds used were determined to be the lowest concentration that resulted in >95% inhibition of visual growth at 48 h. The wt strain BY4741 was used as a standard control throughout the experiments.

Sequence similarity searches. A search for sequences of the GT-IS and GT-IR genes identified in our screen that were homologous to human genes was performed using the tBLASTN program from NCBI.

RESULTS

In our primary screen, we identified 56 strains (44 with GT-IS and 12 with GT-IR) exhibiting changes at the MIC of GT. Among those, 43 mutants (41 with GT-IS and 2 with GT-IR) exhibited either defective growth ($n = 39$) or a less-than-fourfold change in GT susceptibility on retesting ($n = 4$) and were eliminated from further characterization. Thus, our screens led to the identification of 10 GT-IR strains and 3 GT-IS strains, comprising 0.27% of the total number of mutants screened (Tables 1 and 2). The MIC of GT was inoculum and medium independent for all GT-IR and GT-IS strains (data not shown).

Specifically, we found that GT-IR was associated with the disruption of genes acting as general metabolism regulators (*OPI1*, *SNF1*, *IFA38*) and was implicated in mitochondrial function (*RTG2*), DNA damage repair (*RAD18*), and protein vesicular transport (*APL2*). We also identified four genes of unknown function (Table 1). In contrast, the disruption of genes encoding proteins involved in transsulfuration (*CYS3*) and mitochondrial translational elongation (*MEF2*) and one gene of unknown function (*YKL037W*) led to GT-IS (Table 2).

We then assessed the specificity of the genes selected in the primary screens by testing the susceptibility of the GT-IR and GT-IS mutants to conventional antifungal agents acting on different cellular targets, protein synthesis inhibitors (cycloheximide), and oxidative stress (H_2O_2). Importantly, specificity for GT-IR and GT-IS was observed for all mutants tested, as all of them exhibited susceptibility patterns similar to that of the control isogenic wt strain (Tables 1 and 2). Notably, most of the selected GT-IR (6/10) and GT-IS (2/3) mutants were shown to have human homologs (Tables 1 and 2).

DISCUSSION

GT, a mycotoxin with a wide range of immunosuppressive properties, has been recently shown to play an important role in *A. fumigatus* virulence (19, 22, 24). Most studies on the mechanisms of GT action have focused on its proapoptotic effects in mammalian cells (7). Nonetheless, a recent gene expression profiling study of human polymorphonuclear neutrophils exposed to GT demonstrated broad-spectrum changes on the host transcriptosome (8). In addition to its direct immunosuppressive properties, GT also affects other important cellular functions, such as actin cytoskeleton organization (3). In this study, we employed the model fungus *S. cerevisiae* to perform a comprehensive analysis on the molecular mechanisms of GT action. Our high-throughput screens in an *S. cerevisiae* single-gene-deletion library identified an array of novel cellular targets of GT.

We initially found that the deletion of a significant number of genes with distinct functions ($n = 6$) led to GT-IR. Most (3/6, 50%) of these genes with predicted functions encoded proteins involved in general metabolism. In particular, two of

TABLE 1. GT-IR genes and their corresponding functions

Gene	Median MIC[a] (μg/ml)					Radius (mm) of zone of growth inhibition (mean ± SD)[b]	Presence of human homolog	Growth rate (%) (mean ± SD)	Function(s) [reference(s)]
	CAS	AMB	FLC	Cycloheximide	GT				
SNF1	0.007	0.5	16.0	0.025	8	15.0 ± 0.70	+	86 ± 3	General metabolic pathways/biosynthesis, AMP-activated serine/threonine kinase (26)
IFA38	0.007	0.5	16.0	0.025	2	19.7 ± 0.3	+	93 ± 5	General metabolic pathways/biosynthesis, microsomal beta-keto-reductase (1)
OPI1	0.007	0.5	16.0	0.025	2	16.8 ± 2.5	−	100 ± 5	General metabolic pathways/biosynthesis, negative regulator of phospholipid biosynthesis (25)
RTG2	0.007	0.5	16.0	0.025	8	21.2 ± 0.3	+	96 ± 4	Mitochondrial function/transcriptional activator of RTG and TOR pathways (5, 15)
APL2	0.007	0.5	16.0	0.025	8	21.5 ± 0.3	+	99 ± 3	Vesicular transport, protein processing/beta-adaptin (27)
RAD18	0.007	0.5	16.0	0.025	8	21.5 ± 0.7	+	92 ± 4	DNA damage repair/postreplication repair (17, 21)
YGL235W	0.007	0.5	16.0	0.025	8	19.6 ± 3.6	−	101 ± 4	Unknown function/potential Cdc28 substrate
YOR345C	0.007	0.5	16.0	0.025	16	19.1 ± 1.5	−	97 ± 5	Unknown function
YLR456W	0.007	0.5	16.0	0.025	8	19.5 ± 0.7	+	100 ± 3	Unknown function
YGL072C	0.007	0.5	16.0	0.025	16	24.5 ± 0.7	−	93 ± 5	Unknown function

[a] The median MICs (μg/ml) of the BY4741 (wt) strain to CAS, AMB, FLC, cycloheximide, and GT were 0.007, 0.125, 32, 0.025, and 0.5, respectively, as determined by broth microdilution susceptibility testing in both inocula tested (~10^5 and ~10^3 yeast cells/well).
[b] The disk diffusion method showed that the radius of the zone of growth inhibition of the BY4741 strain to H_2O_2 (21.0 ± 0.7 mm) was comparable to those of the GT-IR and GT-IS strains (*P* was not significant for all comparisons).

these genes are involved in lipid biogenesis, including *OPI1*, a negative regulator of lipid biosynthesis (25), and *IFA38*, which encodes a microsomal beta-keto-reductase (1). Another gene in this class, *SNF1*, encodes an AMP-activated serine/threonine protein kinase (26). This latter enzyme is involved in key metabolic processes, such as peroxisome biogenesis, regulation of carbohydrate metabolism, and starvation. Overall, these genes may shed light on the biosynthetic and catabolic pathways of GT metabolism by the mammalian cells. It is reasonable to speculate that the lack of these genes might result in (i) derepression of downstream regulatory genes with a role in detoxification or (ii) reduced uptake/exposure of GT by its molecular targets, leading to GT-IR mutants.

In addition, one of the GT-IR genes encoded the mitochondrial protein RTG2, which is the transcriptional regulator of the evolutionarily conserved TOR pathway and plays a key role in mitochondrial metabolism and aging (15). Importantly, *RTG2* yeast mutants are reported to be resistant to licorine, an alkaloid with inhibitory effects on total DNA and RNA synthesis (5). It has been theorized that in these mutants, the dysfunctional mitochondrial status stimulates overexpression of nuclear genes involved in both nuclear and mitochondrial-DNA replication through a process of retrograde regulation (5). Interestingly, *APL2*, encoding a protein involved in vesicular transport by clathrin-dependent Golgi protein sorting, was associated with GT-IR (27). Although uptake mechanisms of GT and other epipolythiodioxopiperazines by mammalian target cells are well described, the manner in which these compounds are exported by such cells or from the fungi that produce them has not been reported (7). Hence, vesicular transport and vacuole protein sorting may involve specific organelles required for GT metabolism in yeast cells. To that end, it is plausible that GT may undergo clathrin-mediated coating upon cellular uptake. Finally, the deletion of *RAD18*, an E3 ubiquitin ligase gene required for postreplicational DNA, was found

TABLE 2. GT-IS genes and their corresponding functions

Gene	Median MIC[a] (μg/ml)					Radius (mm) of zone of growth inhibition (mean ± SD)[b]	Presence of human homolog	Growth rate (%) (mean ± SD)	Gene function(s) (reference)
	CAS	AMB	FLC	Cycloheximide	GT				
CYS3	0.028	0.25	32.00	0.025	0.06	22.7 ± 0.3	+	94 ± 6	General metabolic pathways, biosynthesis/cystathionine gamma-lyase (18)
MEF2	0.028	0.25	32.00	0.025	0.06	20.9 ± 0.1	+	105 ± 3	Mitochondrial function/transcriptional elongation factor (20)
YKL037W	0.028	0.25	32.00	0.025	0.125	21.8 ± 0.2	−	101 ± 4	Unknown function

[a] The median MICs (μg/ml) of the BY4741 (wt) strain to CAS, AMB, FLC, cycloheximide, and GT were 0.007, 0.125, 32, 0.025, and 0.5, respectively, as determined by broth microdilution susceptibility testing in both inocula tested (~10^5 and ~10^3 yeast cells/well).
[b] The disk diffusion method showed that the radius of the zone of growth inhibition of the BY4741 strain to H_2O_2 (21.0 ± 0.7 mm) was comparable to those of the GT-IR and GT-IS strains (*P* was not significant for all comparisons).

to confer GT-IR (17). Notably, yeast *RAD18* mutants were selectively tolerant of the anticancer agent camptothecin, a topoisomerase I inhibitor that induces double-strand breaks by blocking replication (21). Finally, because DNA damage and nuclear membrane shape are key molecular events during the early stages of apoptosis, these results are in line with the proapoptotic effects of GT in mammalian cells.

On the other hand, a lower number of strains were found to be GT-IS. Hence, we found that the deletion of the evolutionarily conserved gene (*CYS3*) encoding general metabolism and detoxification has a key role in the survival of yeast cells upon exposure to GT. In particular, CYS3 is the terminal enzyme in the transsulfuration pathway for cysteine biosynthesis and is likely required for protection against oxidative stress (18). Because of the presence of a disulfide bridge in the GT molecule, transsulfuration could be a putative pathway involved in GT metabolism. Importantly, *CYS3* was recently identified in a similar high-throughput screening of *S. cerevisiae* deletion strains with increased susceptibility to an inhibitor of angiogenesis (6). We additionally found that the deletion of *MEF2*, a gene encoding translational elongation of mitochondrion, confers GT-IS (20). Because mitochondria are the key organelles of the apoptotic cell machinery, homologous genes are expected to have a functional role in the effects of GT on eukaryotes.

It is important to emphasize that because our screen was performed with a library of *S. cerevisiae* deletion strains representing only nonessential genes, we cannot rule out the possibility that the most relevant *S. cerevisiae* genes associated with GT-IS or GT-IR are essential genes. Likewise, some of the initially identified GT-IS strains that were subsequently eliminated in our study due to defective growth may also carry genes encoding putative GT cellular targets. Furthermore, in view of the considerable differences in gene ontology across phylogeny, we cannot preclude the possibility that the gene functions in some GT targets are different between *S. cerevisiae*, pathogenic fungi, and mammalian cells. Nevertheless, because the majority of the corresponding genes have human homologs, the characterization of the mechanisms of GT-IS and GT-IR in these mutants could improve our understanding of GT cytotoxicity in humans. To that end, studying the effects of GT in mammalian cells lines by using compounds that can mimic the effects of gene deletions and/or by implementing the RNA interference technology to downregulate the function of individual GT-IR and GT-IS genes identified through our screen in the *Saccharomyces* library could elucidate the molecular mechanisms of GT action in humans. Importantly, recent studies highlight the potential of GT as an anticancer agent because of its unique mechanisms of cytotoxicity (7). Therefore, a better understanding of the molecular targets of GT action may also lead to advances in this area of research.

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