Reduced virulence of *Candida albicans* mutants lacking the *GNA1* gene encoding glucosamine-6-phosphate acetyltransferase

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The yeast *GNA1* gene encodes glucosamine-6-phosphate acetyltransferase which catalyses the reaction of glucosamine 6-phosphate with acetyl-CoA to form *N*-acetylglucosamine 6-phosphate, a fundamental precursor in UDP-*N*-acetylglucosamine biosynthesis. *Candida albicans* mutants lacking *GNA1* were viable in the presence of *N*-acetylglucosamine. To confirm the physiological importance of *C. albicans* *GNA1*, the virulence of a *C. albicans* *gna1*Δ null mutant was examined in a mouse model of candidiasis. When injected intravenously into mice, the virulence of the *C. albicans* *gna1*Δ null mutant was significantly attenuated. The reduced virulence appeared to be the result of rapid clearance from host tissue. These data suggest that *C. albicans* *GNA1* is required for survival of the fungus in host animals, probably because an insufficient level of *N*-acetylglucosamine is available from the host tissues.

Keywords: *GNA1*, glucosamine-6-phosphate acetyltransferase, antifungal target, *Candida albicans*, virulence

INTRODUCTION

Candida albicans is an important pathogen causing deep mycosis in immunocompromised patients (Odds, 1987). In addition, *C. albicans* cells undergo morphological conversion between yeast and hyphal forms (Gow & Gooday, 1987; Shepherd, 1985). The ability to form hyphae seems to play a role in virulence (Odds, 1994).

UDP-*N*-acetylglucosamine (UDP-GlcNAc) is an essential precursor of peptidoglycan and lipid A in bacteria (Holtje & Schwarz, 1985; Park, 1987; Raetz, 1987), and of chitin and glycoproteins in yeast (Cabib *et al.*, 1982; Herscovics & Orlean, 1993). In the yeast *Saccharomyces cerevisiae*, four enzymes are involved in the reaction from fructose 6-phosphate to UDP-GlcNAc: (i) glutamine:fructose-6-phosphate amidotransferase (Watzel & Tanner, 1989; Smith *et al.*, 1996); (ii) glucosamine-6-phosphate acetyltransferase (Mio *et al.*, 1999); (iii) phosphoacetylglucosamine mutase (Hofmann *et al.*, 1994); and (iv) UDP-GlcNAc pyrophosphorylase (Mio *et al.*, 1998). The gene for glutamine:fructose-6-phosphate amidotransferase has

been cloned by complementing the *gcn1* mutation, and its expression is increased several fold by α -factor (Whelan & Ballou, 1975; Watzel & Tanner, 1989). *AGM1*, which suppresses the growth defect caused by a *pgm1 pgm2* double mutation, is a phosphoacetylglucosamine mutase gene (Boles *et al.*, 1994; Hofmann *et al.*, 1994). In previous papers, we reported that *UAP1* and *GNA1* genes encode UDP-GlcNAc pyrophosphorylase and glucosamine-6-phosphate acetyltransferase, respectively (Mio *et al.*, 1998, 1999). The genes for UDP-GlcNAc biosynthesis are conserved between *S. cerevisiae* and *C. albicans*. In addition, disruption of each of the *S. cerevisiae* genes *GFA1* (Watzel & Tanner, 1989), *GNA1*, *AGM1* and *UAP1* was lethal, suggesting that enzymes involved in UDP-GlcNAc biosynthesis are potential targets for antifungal agents.

On the other hand, a GlcNAc catabolic pathway has been reported in *C. albicans*. GlcNAc can be transported into the cells by GlcNAc permease and converted to fructose 6-phosphate by the sequential action of GlcNAc kinase, GlcNAc-6-phosphate deacetylase and glucosamine-6-phosphate deaminase (Singh & Datta, 1979; Gopal *et al.*, 1982; Datta *et al.*, 1989). Therefore, *C. albicans* *gna1* null mutants should grow in the presence of GlcNAc. In this work, we obtained *C. albicans* heterozygous and homozygous *gna1*Δ null mutants and

Abbreviations: GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine.

characterized their phenotypes. In addition, we show the effect of a mutation in *GNA1* on virulence in a mouse model of candidiasis.

METHODS

***C. albicans* strains.** The *C. albicans* strains used in this study are listed in Table 1.

Disruption of the *C. albicans* *GNA1* gene. Gene disruption was carried out according to the *ura* blaster protocol (Alani *et al.*, 1987). To disrupt the *C. albicans* *GNA1* gene, pGNA1 was constructed by cloning the 450 bp *Bam*HI–*Sma*I fragment of *C. albicans* *GNA1* into pUC19 (Fig. 1a). After digestion of pGNA1 with *Eco*T22I, the resulting DNA fragment was ligated with a 3·8 kb *Bam*HI–*Bgl*II fragment carrying the *hisG*–*URA3*–*hisG* cassette to generate pGNA1U. Thus, *hisG*–*URA3*–*hisG* was inserted into the *Eco*T22I site of *C. albicans* *GNA1*. After pGNA1U had been linearized by digestion with *Bam*HI and *Sma*I, 100 µg of the DNA was transfected into *C. albicans* CAI4 cells by the lithium acetate method (Ito *et al.*, 1983), and CGM1 was obtained as a single *GNA1* allelic disruption by selection of *Ura*⁺ transformants. Before the second round of transformation, the *URA3* gene that had been integrated into the *C. albicans* genome was excised by 5-fluoroorotic acid (Lundblad, 1992). CGM120 was isolated as a *gna1*Δ null mutant after the second round of transformation with the same cassette. The *C. albicans* *URA3* gene was obtained by functional complementation. YPH499 (*MATa ura3 lys2 ade2 trp1 his3 leu2*) was transformed with a *C. albicans* genomic library (Yamada-Okabe *et al.*, 1996) and uracil prototrophs were selected on –*ura* plates. pCaURA3 was constructed by cloning the 3·3 kb *Sty*I–*Pst*I fragment containing the entire *C. albicans* *URA3* into pUC18. After digestion of pCaURA3 with *Pst*I and *Sma*I, the resulting DNA fragment was transformed into CGM12 and CGM120 to integrate it into the *ura3*Δ::*imm434* locus. All *C. albicans* cells were cultured in YPDGlcNAc medium (2% peptone, 1% yeast extract, 2% glucose and 2% GlcNAc) at 30 °C.

Southern blot analysis. Genomic DNA was prepared as described by Kasahara *et al.* (1994). Twenty-five micrograms of genomic DNA that had been digested with *Nsp*V was fractionated by agarose gel electrophoresis, transferred to Nylon membranes and hybridized with the 163 bp *Sma*I–*Nde*I

fragment of *C. albicans* *GNA1*. Hybridization was carried out under stringent conditions in a buffer containing 50 mM sodium phosphate (pH 6·5), 5× SSC, 5× Denhardt's solution, 50% (v/v) formamide, 0·25 mg salmon sperm DNA ml^{−1} and 0·1% (w/v) SDS at 42 °C for 18 h.

Assay for glucosamine-6-phosphate acetyltransferase (EC 2·3·1·4). Glucosamine-6-phosphate acetyltransferase activity was determined by measurement of CoA produced. Since CoA reacts with 2-nitrobenzoic acid and releases 4-nitrothiophenolate (Gehring *et al.*, 1996; Riddles *et al.*, 1983), an assay was performed in a 50 µl reaction mixture containing 50 mM Tris/HCl (pH 7·5), 5 mM MgCl₂, 150 µM glucosamine 6-phosphate, 150 µM acetyl-CoA, 10% (v/v) glycerol and 70 µg of crude extract obtained from the mutants. After incubation at 37 °C for 20 min, the reaction was terminated by adding a solution containing 50 mM Tris/HCl (pH 7·5) and 6·4 M guanidine hydrochloride, and then 50 µl of a solution containing 50 mM Tris/HCl (pH 7·5), 1 mM EDTA and 20 µM 2-nitrobenzoic acid. The amount of CoA produced by glucosamine-6-phosphate acetyltransferase was estimated from that of 4-nitrothiophenolate by measuring the absorbance at 412 nm.

Systemic infection of mice. Stationary-phase *C. albicans* cells were harvested from the YPDGlcNAc cultures. The cells were washed with sterile distilled water, suspended in saline and counted with a haemocytometer. Cell suspensions (0·2 ml) containing 1 × 10⁵, 1 × 10⁶ and 1 × 10⁷ cells were then injected intravenously into mice. Each test strain was injected into five mice.

Quantification of *C. albicans* in infected tissue. Cell suspensions (0·2 ml) containing 1 × 10⁵, 1 × 10⁶ and 1 × 10⁷ cells were injected intravenously into mice. The number of *C. albicans* cells in the kidney and liver was examined 3 d after infection.

RESULTS

Disruption of the *C. albicans* *GNA1* gene

S. cerevisiae *GNA1* (*ScGNA1*) is an essential gene in this yeast. Furthermore, the addition of GlcNAc did not suppress the growth defect of *ScGNA1*-deficient cells (data not shown). Because *S. cerevisiae* does not carry

Table 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Reference
CAI4	CAF2	<i>ura3</i> Δ:: <i>imm434</i> / <i>ura3</i> Δ:: <i>imm434</i>	Fonzi & Irwin (1993)
CGM1	CAI4	<i>ura3</i> Δ:: <i>imm434</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>GNA1</i> / <i>gna1</i> Δ:: <i>hisG</i> – <i>URA3</i> – <i>hisG</i>	This work
CGM12	CGM1	<i>ura3</i> Δ:: <i>imm434</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>GNA1</i> / <i>gna1</i> Δ:: <i>hisG</i>	This work
CGM120	CGM12	<i>ura3</i> Δ:: <i>imm434</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>gna1</i> Δ:: <i>hisG</i> / <i>gna1</i> Δ:: <i>hisG</i> – <i>URA3</i> – <i>hisG</i>	This work
CGM1210	CGM121	<i>ura3</i> Δ:: <i>imm434</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>gna1</i> Δ:: <i>hisG</i> / <i>gna1</i> Δ:: <i>hisG</i>	This work
CAF2	SC5314	<i>URA3</i> / <i>ura3</i> Δ:: <i>imm434</i>	Fonzi & Irwin (1993)
CGM12-1	CGM12	<i>URA3</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>GNA1</i> / <i>gna1</i> Δ:: <i>hisG</i>	This work
CGM1210-1	CGM1210	<i>URA3</i> / <i>ura3</i> Δ:: <i>imm434</i> , <i>gna1</i> Δ:: <i>hisG</i> / <i>gna1</i> Δ:: <i>hisG</i>	This work

Fig. 1. Disruption of *GNA1*. (a) Strategy for disruption of *GNA1* and the DNA used for the probe. Restriction endonuclease sites and nucleotide positions, which are counted from the *NspV* site present in the 5' noncoding region of *GNA1*, are indicated. (b) Integration of *hisG* sequences into *GNA1* alleles was confirmed by Southern blotting. Genomic DNA (25 µg) that had been digested with *NspV* was fractionated by agarose gel electrophoresis, transferred to Nylon membranes and hybridized with the 163 bp *SmaI*-*NdeI* fragment of *GNA1* as a probe. Bands derived from the *GNA1* allele, the *gna1*Δ::*hisG-URA3-hisG* allele and the *gna1*Δ::*hisG* allele are indicated by A, B and C, respectively. Lanes: 1, CAI4; 2, CGM1; 3, CGM12; 4, CGM120; 5, CGM1210.

the gene for GlcNAc kinase, UDP-GlcNAc is not synthesized in these cells. In contrast, the *C. albicans* *gna1*Δ null mutant should grow in the presence of GlcNAc because GlcNAc kinase exists in *C. albicans*. To test this hypothesis, transformants in the second round of disruption of the *GNA1* locus were selected on plates supplemented with 2% GlcNAc. When 100 transformants were plated onto YPD, 8 out of 100 did not grow. The result of Southern blot analysis revealed that the *hisG-URA3-hisG* module was correctly integrated into the *GNA1* locus and that *URA3* was eliminated by 5-fluoroorotic acid (Fig. 1b). Thus, a null

mutation of *gna1* in *C. albicans* is lethal in the absence of GlcNAc.

To examine the effect of *GNA1* disruption on glucosamine-6-phosphate acetyltransferase, the activity of the enzyme from mutants was measured. When incubated with glucosamine 6-phosphate and acetyl-CoA, the crude extract from wild-type CAI4 produced 38.9 ± 3.9 µM CoA from acetyl-CoA. In contrast, the homozygous *gna1*Δ null mutant (CGM1210-1) showed a large reduction in glucosamine-6-phosphate acetyltransferase activity (2.0 ± 0.4 µM CoA released), and the activity of the heterozygous *gna1*Δ mutant (CGM12-1) retained about 40% of wild-type CAI4 activity (14.2 ± 2.9 µM CoA released), indicating a gene dosage effect.

Effect of *GNA1* disruption on morphology and growth

In *S. cerevisiae*, *ScGNA1*-deficient cells exhibited morphological defects: most of the yeast cells swelled and then often lysed (Mio *et al.*, 1999). The role of *GNA1* on *C. albicans* growth was investigated. CGM1210-1 grew and formed hyphae in YPD + GlcNAc (Fig. 2b), and the growth rate was indistinguishable from that of wild-type (CAF2) or CGM12-1 (the doubling times for CAF2, CGM12-1 and CGM1210-1 were 71, 64 and 73 min, respectively) (Fig. 2a). When CGM1210-1 was cultured on YPD, most of the cells dramatically enlarged, swelled and became defective in cell separation (Fig. 2b). This phenotype was similar to that caused by depletion of *ScGNA1*, suggesting that *C. albicans* *GNA1* is also essential for growth and the synthesis of UDP-GlcNAc.

Next, the ability of CGM1210 to grow on media containing other acetylhexosamines was assessed. CGM1210 grew on *N*-acetylmannosamine (ManNAc) and *N*-acetylgalactosamine (GalNAc) as carbon sources, but the growth was slower than that with GlcNAc (Fig. 3). The addition of glucose significantly decreased growth on ManNAc and GalNAc, whereas there was no effect on GlcNAc (Fig. 3). These data suggest that UDP-GlcNAc can be synthesized from GlcNAc, ManNAc or GalNAc, and that the catabolic pathways of both ManNAc and GalNAc are repressed by glucose. Next, we examined the growth of CGM1210 over a range of GlcNAc concentrations. CGM1210 failed to grow at a GlcNAc concentration less than 10 µM (data not shown). In addition, 20% calf serum did not suppress the growth defect caused by the *gna1*Δ mutation, indicating that the concentration of GlcNAc in calf serum is less than 10 µM.

Effect of disruption of *GNA1* on the virulence of *C. albicans*

To investigate whether *GNA1* was required for *C. albicans* infection, we examined the virulence of mutant strains in a mouse model of systemic candidiasis. All mice infected with 1×10^5 , 1×10^6 or 1×10^7 cells of CGM1210-1 survived (Fig. 4). In contrast, all mice infected with the parental strain CAF2 succumbed to the

Fig. 2. Effects of disruption of *GNA1* on growth and morphology. (a) Effects on growth of wild-type CAF2 (○), CGM12-1 (△) and CGM1210-1 (□). Cells of the indicated strains were cultured in YPD medium in the absence (left) or presence (right) of GlcNAc and cell growth was monitored with a Biophotorecorder (Advantec). (b) Morphological change caused by the disruption of *GNA1*. *C. albicans* cells of CAF2, CGM12-1 and CGM1210-1 were cultured in YPD medium in the presence (upper panels) or absence (lower panels) of GlcNAc and incubated at 30 °C. Photographs were taken after 18 h. Bars, 40 µm (upper panels), 20 µm (lower panels).

Fig. 3. Ability of *gna1*Δ mutants to grow on acetylhexosamines. Cell suspensions (5 µl) containing 1×10^5 cells of CAF4 (wild-type), CGM12 or CGM1210 were spotted on agar plates containing GlcNAc, ManNAc or GalNAc in the absence or presence of glucose (Glc) and incubated at 30 °C. Photographs were taken after 24 h.

Fig. 4. Survival of mice following infection with *C. albicans*. Effects of the disruption of *GNA1* on the virulence of CAF2 (wild-type; ○), CGM12-1 (△) and CGM1210-1 (□). Saline (0.2 ml) containing 1×10^5 (a), 1×10^6 (b) or 1×10^7 (c) cells was injected intravenously into mice. Each test strain was injected into five mice.

infection within 2 d, and the extent of mortality of CGM12-1 was half that of CAF2 (Fig. 4).

The number of *C. albicans* cells in host tissues infected with mutant strains was determined (Table 2). No cells were recovered from the mice infected with CGM1210-1, indicating that CGM1210-1 was rapidly cleared from the kidney and liver. In addition, the recovery of CGM12-1 was significantly lower than that of the wild-type, demonstrating that the virulence of mutant strains correlated with the level of glucosamine-6-phosphate

Table 2. Recovery of *C. albicans* from infected tissues

Challenge dose	<i>C. albicans</i> strain	$\log_{10}$ C.f.u. per mouse (mean $\pm$ SD)	
		Kidney	Liver
1×10^7	CAF2	6.07 ± 0.09	4.21 ± 0.23
	CGM12-1	3.94 ± 0.43	3.41 ± 0.27
	CGM1210-1	0†	$1.45 \pm 0.21^*$
1×10^6	CAF2	4.29 ± 0.20	2.70 ± 0.16
	CGM12-1	$2.99 \pm 0.55^*$	2.38 ± 0.46
	CGM1210-1	0†	0†
1×10^5	CAF2	3.01 ± 0.30	1.70 ± 0.17
	CGM12-1	$2.30 \pm 0.01^*$	$1.78 \pm 0.02^*$
	CGM1210-1	0†	0†

* Some mice cleared infection.

† All mice cleared infection.

acetyltransferase activity (Table 2). These data suggest that *GNA1* is essential for *C. albicans* virulence.

DISCUSSION

In this work, we investigated the physiological role of *GNA1* through characterization of the phenotypes of *C. albicans* mutants. We found that the *C. albicans gna1Δ* null mutant grew in the presence of GlcNAc, whereas *ScGNA1*-deficient *S. cerevisiae* cells did not. This indicates that GlcNAc is converted to UDP-GlcNAc by the sequential actions of GlcNAc kinase, phospho-GlcNAc mutase and UDP-GlcNAc pyrophosphorylase. ManNAc and GalNAc also suppressed the growth defect caused by the *GNA1* disruption. GlcNAc kinase and ManNAc-2-epimerase activities have been reported in *C. albicans* (Biswas *et al.*, 1979). These facts suggest that ManNAc and GalNAc are converted to GlcNAc by each respective epimerase.

The avirulence of the homozygous *gna1Δ* null mutant indicates that *GNA1* is important for the virulence of *C. albicans*. In addition, the heterozygous *gnaΔ* mutant displayed an intermediate virulence phenotype, suggesting that the reduced virulence of *gna1Δ* mutants is associated with the loss of glucosamine-6-phosphate acetyltransferase activity.

The homozygous *gna1Δ* null mutant is not defective in growth rate and hyphal formation in the presence of enough GlcNAc. In addition, the calcofluor susceptibility of the mutants is similar to that of the wild-type, suggesting that the mutants synthesize chitin from exogenous GlcNAc in an amount sufficient for growth (data not shown). These facts suggest that the homozygous *gnaΔ* null mutant does not survive in host animals because of an insufficient level of GlcNAc in the tissues.

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