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

Toshiyuki Mio, Michiko Kokado, Mikio Arisawa and Hisafumi Yamada-Okabe

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).

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 gna1Δ null mutant was examined in a mouse model of candidiasis. When injected intravenously into mice, the virulence of the C. albicans gna1Δ 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

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

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 gna1Δ null mutant was examined in a mouse model of candidiasis. When injected intravenously into mice, the virulence of the C. albicans gna1Δ 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

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

the reaction from fructose 6-phosphate to UDP-GlcNAc: (i) glutamine:fructose-6-phosphate amidotransferase (Watzele & 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; Watzele & 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 (Watzele & 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 gna1Δ null mutants and...
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 BamH–Smal fragment of C. albicans GNA1 into pUC19 (Fig. 1a). After digestion of pGNA1 with EcoT22I, the resulting DNA fragment was ligated with a 3/8 kb BamH–BglII fragment carrying the bisG–URA3–bisG cassette to generate pGNA1U. Thus, bisG–URA3–bisG was inserted into the EcoT22I site of C. albicans GNA1. After pGNA1U had been linearized by digestion with BamH and Smal, 100 µg of the DNA was transfected into C. albicans CAH4 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 gna1A null mutant after the second round of transformation with the same cassette. The C. albicans URA3 gene was obtained by functional complementation. YPH499 (MATα ura3 ade2 trp1 his3 lur1) was transformed with a C. albicans genomic library (Yamada-Okabe et al., 1996) and ura3· prototrophs were selected on −ura plates. pCaURA3 was constructed by cloning the 3.3 kb StyI–PstI fragment containing the entire C. albicans URA3 into pUC18. After digestion of pCaURA3 with PstI and Smal, the resulting DNA fragment was transformed into CGM12 and CGM120 to integrate it into the ura3A::imm434 locus. All C. albicans cells were cultured in YPDGlcnac medium (2% peptone, 1% yeast extract, 2% glucose and 2% GlnAc) 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 NspV was fractionated by agarose gel electrophoresis, transferred to Nylon membranes and hybridized with the 163 bp Smal–Ndel 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 (Gehrting 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 64 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 × 109, 1 × 108 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 × 109, 1 × 108 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>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAI4</td>
<td>CAF2</td>
<td>ura3Δ::imm434/ura3Δ::imm434, GNA1/gna1Δ::hisG–URA3–hisG</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CGM1</td>
<td>CAI4</td>
<td>ura3Δ::imm434/ura3Δ::imm434, GNA1/gna1Δ::hisG–URA3–hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CGM12</td>
<td>CGM1</td>
<td>ura3Δ::imm434/ura3Δ::imm434, GNA1/gna1Δ::hisG–URA3–hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CGM120</td>
<td>CGM12</td>
<td>ura3Δ::imm434/ura3Δ::imm434, gna1Δ::hisG/gna1Δ::hisG–URA3–hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CGM1210</td>
<td>CGM120</td>
<td>ura3Δ::imm434/ura3Δ::imm434, gna1Δ::hisG/gna1Δ::hisG–URA3–hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CAF2</td>
<td>SC5314</td>
<td>URA3/ura3Δ::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CGM12-1</td>
<td>CGM12</td>
<td>URA3/ura3Δ::imm434, GNA1/gna1Δ::hisG</td>
<td>This work</td>
</tr>
<tr>
<td>CGM1210-1</td>
<td>CGM120</td>
<td>URA3/ura3Δ::imm434, gna1Δ::hisG/gna1Δ::hisG</td>
<td>This work</td>
</tr>
</tbody>
</table>
the gene for GlcNAc kinase, UDP-GlcNAc is not synthesized in these cells. In contrast, the \textit{C. albicans} gna1\textsuperscript{Δ} null mutant should grow in the presence of GlcNAc because GlcNAc kinase exists in \textit{C. albicans}.

To test this hypothesis, transformants in the second round of disruption of the \textit{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 \textit{hisG-URA3-hisG} module was correctly integrated into the \textit{GNA1} locus and that \textit{URA3} was eliminated by 5-fluoroorotic acid (Fig. 1b). Thus, a null mutation of \textit{gna1} in \textit{C. albicans} is lethal in the absence of GlcNAc.

To examine the effect of \textit{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 \(\mu\)M CoA from acetyl-CoA. In contrast, the homozygous \textit{gna1\textsuperscript{Δ}} null mutant (CGM1210-1) showed a large reduction in glucosamine-6-phosphate acetyltransferase activity (2.0 ± 0.4 \(\mu\)M CoA released), and the activity of the heterozygous \textit{gna1A} mutant (CGM12-1) retained about 40\% of wild-type CAI4 activity (14.2 ± 2.9 \(\mu\)M CoA released), indicating a gene dosage effect.

**Effect of \textit{GNA1} disruption on morphology and growth**

In \textit{S. cerevisiae}, \textit{ScGNA1}-deficient cells exhibited morphological defects: most of the yeast cells swelled and then often lysed (Mio \textit{et al}., 1999). The role of \textit{GNA1} on \textit{C. albicans} growth was investigated. CMG1210-1 grew and formed hyphae in YPD + GlcNAc (Fig. 2b), and the growth rate was indistinguishable from that of wild-type (CAF2) or CMG12-1 (the doubling times for CAF2, CMG12-1 and CMG1210-1 were 71, 64 and 73 min, respectively) (Fig. 2a). When CMG1210-1 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 \textit{ScGNA1}, suggesting that \textit{C. albicans} \textit{GNA1} is also essential for growth and the synthesis of UDP-GlcNAc.

Next, the ability of CMG1210 to grow on media containing other acetylated sugars was assessed. CMG1210 grew on \textit{N}-acetylmannosamine (ManNAc) and \textit{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 CMG1210 over a range of GlcNAc concentrations. CMG1210 failed to grow at a GlcNAc concentration less than 10 \(\mu\)M (data not shown). In addition, 20\% calf serum did not suppress the growth defect caused by the \textit{gna1A} mutation, indicating that the concentration of GlcNAc in calf serum is less than 10 \(\mu\)M.

**Effect of disruption of \textit{GNA1} on the virulence of \textit{C. albicans}**

To investigate whether \textit{GNA1} was required for \textit{C. albicans} infection, we examined the virulence of mutant strains in a mouse model of systemic candidiasis. All mice infected with \(1 \times 10^5\), \(1 \times 10^6\) or \(1 \times 10^7\) cells of CMG1210-1 survived (Fig. 4). In contrast, all mice infected with the parental strain CAF2 succumbed to the disease within 7 days.
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 gna1Δ mutants to grow on acetylhexosamines. Cell suspensions (5 µl) containing 1 × 10⁵ cells of CAI4 (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.
The number of C. albicans CGM12-1 was half that of CAF2 (Fig. 4). Infection within 2 d, and the extent of mortality of C. albicans into five mice. Each test strain was injected intravenously into mice. Each test strain was injected into five mice.

**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 $1 \times 10^5$ (a), $1 \times 10^6$ (b) or $1 \times 10^7$ (c) cells was injected intravenously into mice. Each test strain was injected into five mice.

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

<table>
<thead>
<tr>
<th>Challenge dose</th>
<th>C. albicans strain</th>
<th>log_{10} C.f.u. per mouse (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
</tr>
<tr>
<td>$1 \times 10^7$</td>
<td>CAF2</td>
<td>$6.07 ± 0.09$</td>
</tr>
<tr>
<td></td>
<td>CGM12-1</td>
<td>$3.94 ± 0.43$</td>
</tr>
<tr>
<td></td>
<td>CGM1210-1</td>
<td>0†</td>
</tr>
<tr>
<td>$1 \times 10^6$</td>
<td>CAF2</td>
<td>$4.29 ± 0.20$</td>
</tr>
<tr>
<td></td>
<td>CGM12-1</td>
<td>$2.99 ± 0.55$*</td>
</tr>
<tr>
<td></td>
<td>CGM1210-1</td>
<td>0†</td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>CAF2</td>
<td>$3.01 ± 0.30$</td>
</tr>
<tr>
<td></td>
<td>CGM12-1</td>
<td>$2.30 ± 0.01$*</td>
</tr>
<tr>
<td></td>
<td>CGM1210-1</td>
<td>0†</td>
</tr>
</tbody>
</table>

* 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 (Biswa 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 gna1Δ 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 gna1Δ null mutant does not survive in host animals because of an insufficient level of GlcNAc in the tissues.
ACKNOWLEDGEMENTS

We thank S. B. Miwa for reading the manuscript and T. Takahashi for assisting with the experiments.

REFERENCES


Received 20 December 1999; revised 2 March 2000; accepted 16 March 2000.