

N-Acetylglucosamine (GlcNAc) Induction of Hyphal Morphogenesis and Transcriptional Responses in *Candida albicans* Are Not Dependent on Its Metabolism*

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N-Acetylglucosamine (GlcNAc) stimulates important signaling pathways in a wide range of organisms. In the human fungal pathogen *Candida albicans*, GlcNAc stimulates hyphal cell morphogenesis, virulence genes, and the genes needed to catabolize GlcNAc. Previous studies on the GlcNAc transporter (*NGT1*) indicated that GlcNAc has to be internalized to induce signaling. Therefore, the role of GlcNAc catabolism was examined by deleting the genes required to phosphorylate, deacetylate, and deaminate GlcNAc to convert it to fructose-6-PO₄ (*HXK1*, *NAG1*, and *DAC1*). As expected, the mutants failed to utilize GlcNAc. Surprisingly, GlcNAc inhibited the growth of the *nag1Δ* and *dac1Δ* mutants in the presence of other sugars, suggesting that excess GlcNAc-6-PO₄ is deleterious. Interestingly, both *hvk1Δ* and an *hvk1Δ nag1Δ dac1Δ* triple mutant could be efficiently stimulated by GlcNAc to form hyphae. These mutants could also be stimulated to express GlcNAc-regulated genes. Because GlcNAc must be phosphorylated by Hxk1 to be catabolized, and also for it to enter the anabolic pathways that form chitin, *N*-linked glycosylation, and glycosylphosphatidylinositol anchors, the mutant phenotypes indicate that GlcNAc metabolism is not needed to induce signaling in *C. albicans*. Thus, these studies in *C. albicans* reveal a novel role for GlcNAc in cell signaling that may also regulate critical pathways in other organisms.

N-Acetylglucosamine (GlcNAc) is an interesting molecule because it plays important roles in both cell structure and cell signaling. GlcNAc contributes significantly to cell-surface structure in a very wide range of organisms; it is a key component of the bacterial cell wall peptidoglycan layer, fungal cell wall chitin, and the extracellular matrix glycosaminoglycans in animal cells. Eukaryotic cells also use GlcNAc to modify cell-surface proteins by *N*-linked glycosylation and in the formation of glycosylphosphatidylinositol anchors. Additionally, GlcNAc is widely used as a signaling molecule. For example, bacterial cells respond to extracellular GlcNAc by altering the production of CURLI fibers that function in biofilm formation (1), and some yeast species are induced by GlcNAc to switch from bud-

ding to a filamentous hyphal morphology that promotes invasive growth (2, 3). Animal cells respond to increased GlcNAc by promoting the post-translational attachment of GlcNAc to intracellular proteins, including the transcription factors c-Myc, p53, and NF-κB that play key roles in cellular regulation (4). This type of *O*-linked GlcNAc attachment has been implicated in a range of human diseases, including cancer and diabetes (5).

GlcNAc signaling is under investigation in the human fungal pathogen *Candida albicans* because it is a potent inducer of hyphal growth, whereas other sugars are not (2). GlcNAc induces two sets of responses that have significance for understanding the mechanisms of pathogenesis. One pathway stimulates *C. albicans* to switch from budding to hyphal growth and to induce expression of virulence genes. These responses are activated by stimulation of adenylyl cyclase and increased cAMP signaling (2, 6–8). Activation of cAMP signaling by GlcNAc also promotes *C. albicans* cells to undergo an epigenetic switch from the white phase morphology to a distinct state known as the opaque phase that is thought to be better suited for mucosal infections (9). This is consistent with the expected presence of GlcNAc in the gastrointestinal tract mucosa as the result of cell wall remodeling by the endogenous bacterial population. GlcNAc also activates a pathway that is independent of cAMP to induce the expression of the genes needed to catabolize GlcNAc (8, 10, 11). The signaling pathways induced by GlcNAc in *C. albicans* are not yet defined well. One reason for this is that the commonly studied model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lack the genes needed to catabolize GlcNAc (12). Also, post-translational modification of proteins by *O*-GlcNAc attachment is not known to occur in *C. albicans* as it does in humans and other metazoans. Thus, *C. albicans* must use distinct GlcNAc signaling strategies whose identification may help to identify novel signaling pathways in metazoans and other organisms.

Previous studies indicated that GlcNAc must enter *C. albicans* cells to induce signaling. Transport of GlcNAc into *C. albicans* is mediated by a recently discovered plasma membrane transporter, Ngt1, which represents the first eukaryotic GlcNAc transporter to be identified (12). Ngt1 appears to specifically transport GlcNAc, and not other related sugars. Mutant cells lacking Ngt1 (*ngt1*) showed greatly reduced GlcNAc uptake and required about 1,000-fold higher levels of GlcNAc to induce signaling (12). This is similar to the results for galactose-induced signaling in *S. cerevisiae*, which is activated by intracellular galactose binding to the cytoplasmic Gal3

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GlcNAc-induced Signaling in *C. albicans*

protein that then shuttles to the nucleus to regulate the Gal4 transcriptional regulator (13, 14). It is not known how GlcNAc is sensed and whether signaling requires its catabolism, which would release acetate and ammonia in the cell. Therefore, in this study we examined how GlcNAc signaling was affected in cells lacking the proteins that catabolize GlcNAc. The genes that mediate GlcNAc catabolism have been identified previously and are known to encode Hxk1, a kinase that phosphorylates GlcNAc to create GlcNAc-6-PO₄, Dac1, a deacetylase that converts it to glucosamine-6-PO₄, and Nag1, a deaminase that converts it to fructose-6-PO₄ that can then be used in glycolysis (10, 11, 15). These experiments also test whether signaling requires the exogenous GlcNAc to enter the anabolic pathways that form chitin, *N*-linked glycosylation, and glycosylphosphatidylinositol anchors on proteins (16). Phosphorylation of exogenous GlcNAc by Hxk1 is needed to create GlcNAc-6-PO₄, which is then converted to UDP-GlcNAc for use as a substrate in the anabolic pathways (16). The results demonstrate that GlcNAc metabolism is not required for induction of hyphal growth or expression of the catabolic genes, thereby identifying a novel role for GlcNAc in cell signaling.

EXPERIMENTAL PROCEDURES

Strains and Media—The genotypes of the *C. albicans* strains used in this study are listed in Table 1. *C. albicans* cells were propagated on rich YPD medium or on synthetic medium (17). The medium was supplemented with 80 mg/liter uridine to permit growth of *ura3* mutants. The ability of cells to grow on different sugars was assayed by spotting dilutions of cells onto solid media agar plates followed by incubation at 37 °C.

Homozygous *dac1Δ/dac1Δ* and *nag1Δ/nag1Δ* deletion mutants that removed the entire open reading frames were constructed in *C. albicans* strain BWP17 (*arg4Δ his1Δ ura3Δ*) using methods described previously (18). In brief, PCR primers containing ~70 bp of sequence homologous to the sequences flanking the open reading frame of *NAG1* or *DAC1* were used to amplify the *ARG4* and the *HIS1* selectable marker genes. Integration of these deletion cassettes at the appropriate sites to delete the corresponding copies of the *NAG1* or *DAC1* genes was verified by PCR using combinations of primers that flanked the integration and also primers that annealed within the introduced cassettes. Complementation of the *nag1Δ* and *dac1Δ* strains was performed by introducing a plasmid carrying one wild-type copy of *NAG1* or *DAC1* into the genome. These plasmids were constructed by PCR amplification of the genomic DNA from 1,000 bp upstream of the initiator ATG to 300 bases downstream of the terminator codon of *NAG1* or *DAC1*. These DNA fragments were then inserted between the *SacI* and *SacII* restriction sites of the *URA3* plasmid pDDB57 (19). The resulting *DAC1* plasmid was linearized in the promoter region by digestion with *PstI* and integrated into the *dac1Δ/dac1Δ* strain AG732 using *URA3* selection to create complemented strains AG762. Similarly, the *NAG1* plasmid was linearized by digestion with *NcoI* and then integrated into a *nag1Δ/nag1Δ* strain to create the complemented strain AG767. To create prototrophic versions of the *dac1Δ/dac1Δ* and *nag1Δ/nag1Δ* mutants, they were transformed with a *URA3*-containing DNA fragment to restore *URA3* at its native locus. The *URA3* frag-

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

Strain	Genotype
BWP17	<i>ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
DIC185	<i>ura3Δ::limm434/URA3 his1::hisG/HIS1 arg4::hisG/ARG4</i>
A G673	<i>NAG1/nag1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G676	<i>nag1::HIS1/nag1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G734	<i>nag1::HIS1/nag1::ARG4 URA3/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G767	<i>nag1::HIS1/nag1::ARG4 NAG1/URA3 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G648	<i>DAC1/dac1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G712	<i>dac1::HIS1/dac1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G732	<i>dac1::HIS1/dac1::ARG4 URA3/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G762	<i>dac1::HIS1/dac1::ARG4 DAC1/URA3 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G697	<i>HXK1/hxk1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G689	<i>hxk1::URA3/hxk1::ARG4 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G736	<i>hxk1::URA3/hxk1::ARG4 ura3Δ::limm434/ura3Δ::limm434 HIS1/his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
SN772	<i>hxk1::URA3/hxk1::ARG4 HXK1/HIS1 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
A G695	Heterozygous h-d strain; [<i>hxk1Δ nag1Δ dac1Δ</i>]:: <i>ARG4/HXK1 NAG1 DAC1 his1::hisG/his1::hisG ura3Δ::limm434/ura3Δ::limm434 arg4::hisG/arg4::hisG</i>
A G692	Homozygous h-d strain; [<i>hxk1Δ nag1Δ</i>]:: <i>ARG4</i>]; <i>hxk1Δ nag1Δ</i>
A G738	<i>HIS⁺</i> homozygous h-d strain; [<i>hxk1 nag1 dac1</i>]:: <i>ARG4</i>]; <i>hxk1 nag1 dac1Δ</i>]; <i>URA3 HIS1/his1::hisG/his1::hisG ura3Δ::limm434/ura3Δ::limm434 arg4::hisG/arg4::hisG</i>
SN787	Complemented h-d strain; [<i>hxk1Δ nag1Δ dac1Δ</i>]:: <i>ARG4</i>]; <i>hxk1Δ nag1Δ dac1Δ</i>]; <i>URA3 HIS1/his1::hisG/his1::hisG ura3Δ::limm434/ura3Δ::limm434 arg4::hisG/arg4::hisG</i>
SN787	<i>NGT1-GFP::HIS1 ura3Δ::limm434/ura3Δ::limm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
SN791	<i>hxk1Δ strain AG689 plus NGT1-GFP::HIS1</i>
SN794	<i>hxk1Δ nag1Δ dac1Δ strain AG692 plus NGT1-GFP::HIS1</i>
180	<i>cph1::HIS1/cph1::LEU2 his1/his1 leu2/leu2 arg4/arg4</i>
CJN305	<i>cph1::UAIU/cph1::URA3 his1::hisG/his1::hisG leu2/leu2 arg4/arg4</i>
1112	<i>mck1::HIS1/mck1::LEU2 his1/his1 leu2/leu2 arg4/arg4</i>
544	<i>cek1::HIS1/cek1::LEU2 his1/his1 leu2/leu2 arg4/arg4</i>
060	<i>efg1::HIS1/efg1::LEU2 his1/his1 leu2/leu2 arg4/arg4</i>
SN250	<i>LEU2/leu2 HIS1/his1 arg4/arg4</i>

ments were liberated from plasmid pBSK-*URA3* (20) by digestion with restriction enzymes PstI and NotI.

For reasons that are unclear, homozygous deletion mutants lacking *HXK1* or lacking the whole set of contiguous genes *HXK1*, *DAC1*, and *NAG1* could not be isolated using selection with *ARG4* and *HIS1*. Therefore, a similar strategy was used in which the corresponding genes were deleted with *ARG4* and *URA3* to create the *hxx1Δ* mutant AG736 and the h-d mutant (*hxx1Δ nag1Δ dac1Δ*) AG738. Complementing plasmids were then created using a derivative of pDDB57 in which *URA3* was replaced with *HIS1*. The complementing plasmids were linearized by digestion with SphI and integrated into the corresponding deletion mutants using selection for *HIS1* to create strains SN772 and SN778. Prototrophic versions of the mutants were created by transformation with a DNA fragment containing *HIS1*.

C. albicans strains carrying *NGT1-GFP* strain were created by homologous recombination of GFP sequences into the 3' end of the *NGT1* open reading frame using previously described methods (21). PCR primers containing ~70 bp of sequence homologous to the 3' end of the *NGT1* open reading frame were used to amplify a cassette containing a more photostable version of enhanced GFP (CaGFP-γ) and a *URA3* selectable marker (21). The resulting Ura⁺ colonies from the transformation into *C. albicans* were then screened for GFP-positive cells by fluorescence microscopy and confirmed by PCR.

Microscopy—The ability of the wild-type control strain DIC185 and mutant cells to form hyphae was carried out with cells grown overnight to early log phase in synthetic medium with galactose. The cells were then resuspended in synthetic medium containing 50 mM galactose plus or minus the indicated concentration of GlcNAc and grown for 2 h at 37 °C and then photographed using differential interference contrast microscopy. Induction of Ngt1-GFP was detected in cells that were grown overnight to log phase in synthetic medium containing dextrose, washed, and then resuspended in medium containing dextrose or GlcNAc as indicated. Ngt1-GFP was detected by fluorescence microscopy, and light microscope images were taken with differential interference contrast optics. Images were captured using an Olympus BH2 microscope equipped with a Zeiss AxioCam digital camera. Fluorescent cell signals were quantified using AxioVision software.

Analysis of mRNA Levels Using Real Time Quantitative RT-PCR (qRT-PCR)⁴—Cells were grown overnight to log phase, washed, and then incubated in synthetic medium containing 50 mM dextrose or GlcNAc for 2 h, and the cell pellets were then frozen at -80 °C. RNA was extracted from a pellet of about 3 × 10⁸ cells using a RiboPure-Yeast RNA isolation kit (Ambion). RNA samples were tested by PCR to confirm the absence of DNA contamination. cDNA was then synthesized from total RNA using an oligo(dT) primer (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). The cDNA samples were treated with RNase A (New England Biolabs) and purified using a PCR cleanup column (Qiagen). The cDNA samples were then used as templates for qRT-PCR in a Mastercycler EP Realplex2

(Eppendorf). The reaction mix included 2× iQ SYBR Green Supermix (Bio-Rad), 1 μl of first-strand cDNA reaction mixture, and 0.1 μM of primers per 10-μl reaction. The amplification program was initiated with a 95 °C denaturation step for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. Raw data were analyzed using Mastercycler Realplex2 software analysis module ($\Delta\Delta C_T$ method) to determine the relative differences in gene expression, which were normalized to the level of *ACT1* mRNA. The specificity of the reaction products was assessed by melting curve analysis at the end of the qPCR program. The results reported represent the average of at least three independent assays each done in triplicate. Where indicated, the results include the analysis of two independent RNA preparations from independently derived mutant strains. The following primers used for qPCR analysis were designed using Primer3 software and ordered from Invitrogen: *ACT1-F* 5'-TCC-AGAAGCTTTGTTTCAGACCAGC-3' and *ACT1-R* 5'-TGCAT-ACGTTTCAGCAATACCTGGG-3'; *NGT1-F* 5'-TCGTGCCAA-AATTGGTTGGGCT-3' and *NGT1-R* 5'-TGGACATGGCTC-CCAATACCCA-3'; *HXK1-F* 5'-TGTGAGCTCTCGTTGTT-TGG-3' and *HXK1-R* 5'-TCAATCCGGCAAGTACCTC-3'; *NAG1-F* 5'-GAAGCGGGATCATCAAGAAA-3' and *NAG1-R* 5'-TGGCAATTTCTGCTGAGTTG-3'; *DAC1-F* 5'-CGTTG-CCGCAATGTGTGCGT-3' and *DAC1-R* 5'-GACGACTGG-ACTTCACGTCCCA-3'; *GIG1-F* 5'-GCAAACCCACCC-ACTTCACCA-3' and *GIG1-R* 5'-TGTTTGTCTGCTGATC-GAGCA-3'; *GAL10-F* 5'-AGGAGCAAACAACCTTGCATGG-TGG-3' and *GAL10-R* 5'-GCTTCAAGCTCACCTGGGA-ACC-3'; and *PMA1-F* 5'-TTGCCAAGATTGTGGGGTAT-3' and *PMA1-R* 5'-GACCAGAATGGACCTTGAGC-3'.

RESULTS

Deletion of *HXK1*, *NAG1*, and *DAC1*—Previous studies identified the *C. albicans* *HXK1*, *NAG1*, and *DAC1* genes that encode the enzymes needed to catabolize GlcNAc (10, 11, 22). Although mutants lacking these genes were constructed previously, there are now concerns regarding the approaches used for recycling of the *URA*-selectable marker to delete both copies of each gene in the diploid *C. albicans* genome and also for the use of control strains carrying a reintroduced copy of the deleted gene (23, 24). Therefore, as described under "Experimental Procedures," we constructed new deletion mutants using the auxotrophic strain BWP17 (*arg4Δ his1Δ ura3Δ*) (18). In addition to creating individual *hxx1Δ*, *nag1Δ*, and *dac1Δ* mutants, we also made a triple mutant. All three genes could be deleted in one step because they are clustered on chromosome 6. For simplicity, the triple *hxx1Δ nag1Δ dac1Δ* mutant will be referred to as the h-d mutant as it removes the gene cluster from *HXK1* to *DAC1*.

The homozygous deletion mutants were examined for their ability to grow on different carbon sources by spotting dilutions of cells onto solid agar medium (Fig. 1). As expected, all of the mutants failed to grow on medium containing GlcNAc as the sole carbon and energy source. There was no growth on GlcNAc for the mutants even after extended periods of time (1 month). Any minor amount of growth observed on GlcNAc medium was similar to that seen for cells grown on a medium lacking any added sugar (data not shown). Control studies

⁴ The abbreviation used is: qRT-PCR, quantitative RT-PCR.

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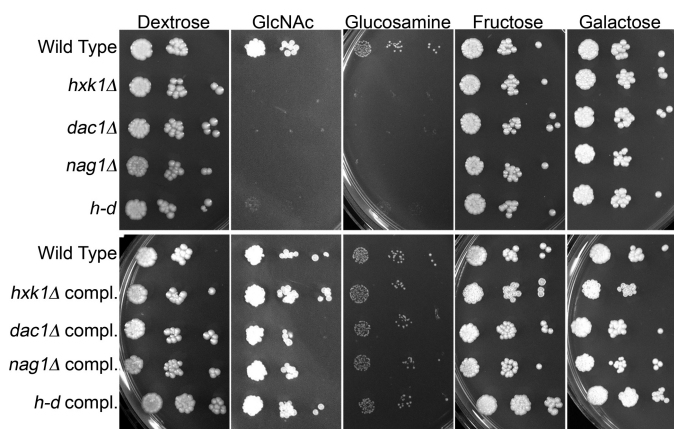


FIGURE 1. Growth of mutant strains on media containing different sugars. Dilutions of a wild-type control and the indicated mutants strains were spotted onto the solid agar media plates containing 50 mM of the indicated sugar and then incubated at 37 °C for 2 days. The strains used in the upper panel are the wild-type control (DIC185), *hxk1Δ* (AG736), *dac1Δ* (AG732), *nag1Δ* (AG734), and a triple *hxk1Δ dac1Δ nag1Δ* mutant referred to as the h-d strain (AG738). The complemented (*compl.*) strains in the lower panel, which carry a wild-type copy of the corresponding gene integrated into the genome of the mutant cells, included *hxk1Δ* + *HXK1* (SN772), *dac1Δ* + *DAC1* (AG762), *nag1Δ* + *NAG1* (AG767), and h-d + *HXK1 NAG1 DAC1* (SN778) strains.

showed that the mutants grew well on media containing dextrose, fructose, and the unrelated sugar galactose but failed to grow on glucosamine medium. It is not clear why the GlcNAc deacetylase mutant (*dac1Δ*) did not grow on glucosamine. However, growth on GlcNAc and glucosamine media was restored in the complemented strains in which one copy of the wild-type gene was reintroduced (Fig. 1, lower panel).

GlcNAc Inhibits Growth of *nag1Δ* and *dac1Δ* Mutants—The response of the mutants to GlcNAc was tested by growing cells in galactose medium and then adding GlcNAc as an inducer. Galactose was selected to provide a source of carbon and energy because it is an unrelated sugar that does not repress induction of GlcNAc catabolic genes, as does dextrose (8, 12). Surprisingly, GlcNAc inhibited the growth of the *nag1Δ* and *dac1Δ* mutants on solid agar plates containing galactose (Fig. 2A). Growth rate studies in liquid showed that addition of 10 mM GlcNAc rapidly blocked growth of the *nag1Δ* and *dac1Δ* mutants even though 50 mM galactose was present as a nutrient source (Fig. 2B). These studies also revealed a very slight delay in the growth of the *hxk1Δ* mutant but no effect on the growth of the h-d mutant. The observation that the *hxk1Δ* and h-d mutants grow well in the presence of GlcNAc indicates that it must be converted to GlcNAc-6-PO₄ to cause the strong inhibitory effects.

Dose-response assays demonstrated that 0.1 mM GlcNAc inhibited the growth of the *nag1Δ* and *dac1Δ* mutants in the presence of 50 mM galactose (Fig. 2C). This inhibitory effect was not specific to galactose medium. GlcNAc also inhibited the growth of the *nag1Δ* and *dac1Δ* mutants in dextrose or fructose media (Fig. 2C), although 100-fold higher concentrations of GlcNAc were required to inhibit growth. These results are consistent with dextrose or fructose having a stronger ability to repress the expression of the GlcNAc transporter (12).

Even though their growth was blocked, the *nag1Δ* and *dac1Δ* mutants maintained a very high degree of viability after 24 h of incubation in galactose plus GlcNAc, indicating GlcNAc inhib-

ited their growth but did not kill them (data not shown). Prolonged incubation of the cells in liquid cultures revealed that the *nag1Δ* mutant eventually recovered after 3 days and grew to high density. This growth was not due to a spontaneous mutation, as the cells were still inhibited when inoculated into fresh medium. In contrast, the *nag1Δ* and *dac1Δ* mutants did not show obvious growth after extended incubation on solid medium agar plates containing galactose plus GlcNAc (Fig. 2A and data not shown).

Similar inhibitory effects of GlcNAc have also been reported for mutant bacteria lacking the orthologs of *NAG1* or *DAC1* (25). In bacteria, the inhibitory effects of GlcNAc could be rapidly reversed by adding uridine to the medium (26). However, addition of uridine did not quickly rescue growth of *C. albicans* *nag1Δ* and *dac1Δ* mutants as it did in bacteria. Uridine did not rescue growth of cells on agar plates, even after extended incubation. Addition of uridine also did not significantly improve the growth of the *nag1Δ* mutant in liquid culture, but it did enable a *C. albicans* *dac1Δ* mutant to resume growth in liquid after a lag of about 3 days. Thus, the effects of uridine on growth of the *C. albicans* *nag1Δ* and *dac1Δ* mutants are distinct from the effects on the analogous *E. coli* mutants.

GlcNAc Catabolism Is Not Needed to Induce Hyphal Growth—The *hxk1Δ* and h-d mutants grew well in the presence of galactose plus GlcNAc, so they were tested for the ability to be induced by GlcNAc to form hyphae. Interestingly, control studies showed that the *hxk1Δ* and the h-d mutants displayed a high basal level of filamentous growth in the absence of GlcNAc (Fig. 3). Deletion of *HXK1* was reported previously to cause filamentous growth as part of a study aimed at defining the mechanisms of epithelial cell colonization (27). Analysis of mutant cells grown in dextrose medium revealed that the filamentous growth was most pronounced in cell cultures grown to high density, whereas the cells maintained at low density were grown primarily as buds (Fig. 3A). Similar results were observed for cells grown in galactose, except that there was a higher degree of abnormal-looking elongated cells in the low density cultures (Fig. 3B), consistent with the expected increase in expression of the GlcNAc transporter in galactose medium. The high level of filamentous growth was specific for the *hxk1Δ* and h-d mutations, as it was not seen in the complemented strains (Fig. 3D). Also, it appears to be due to activation of the hyphal pathway because the cells formed normal looking buds at room temperature, which prevents activation of the hyphal pathway (Fig. 3C). These data suggest that the failure of the *hxk1Δ* and h-d mutants to catabolize GlcNAc released during cell wall remodeling and septation results in it accumulating in the medium and stimulating morphological changes of cells grown to high density. This is supported by the observation that the *hxk1Δ* and h-d mutants also show elevated levels of Ngt1-GFP at high cell density, consistent with the accumulation of GlcNAc in the medium (see below).

The wild-type and mutants were then tested for capacity to be induced by GlcNAc to form hyphae (Fig. 4). Interestingly, the *hxk1Δ* and h-d mutants grown in galactose medium could be induced by GlcNAc to form long filamentous cells with parallel walls, indicative of true hyphae and not just pseudohyphal cells. Dose-response assays showed that the mutants could be

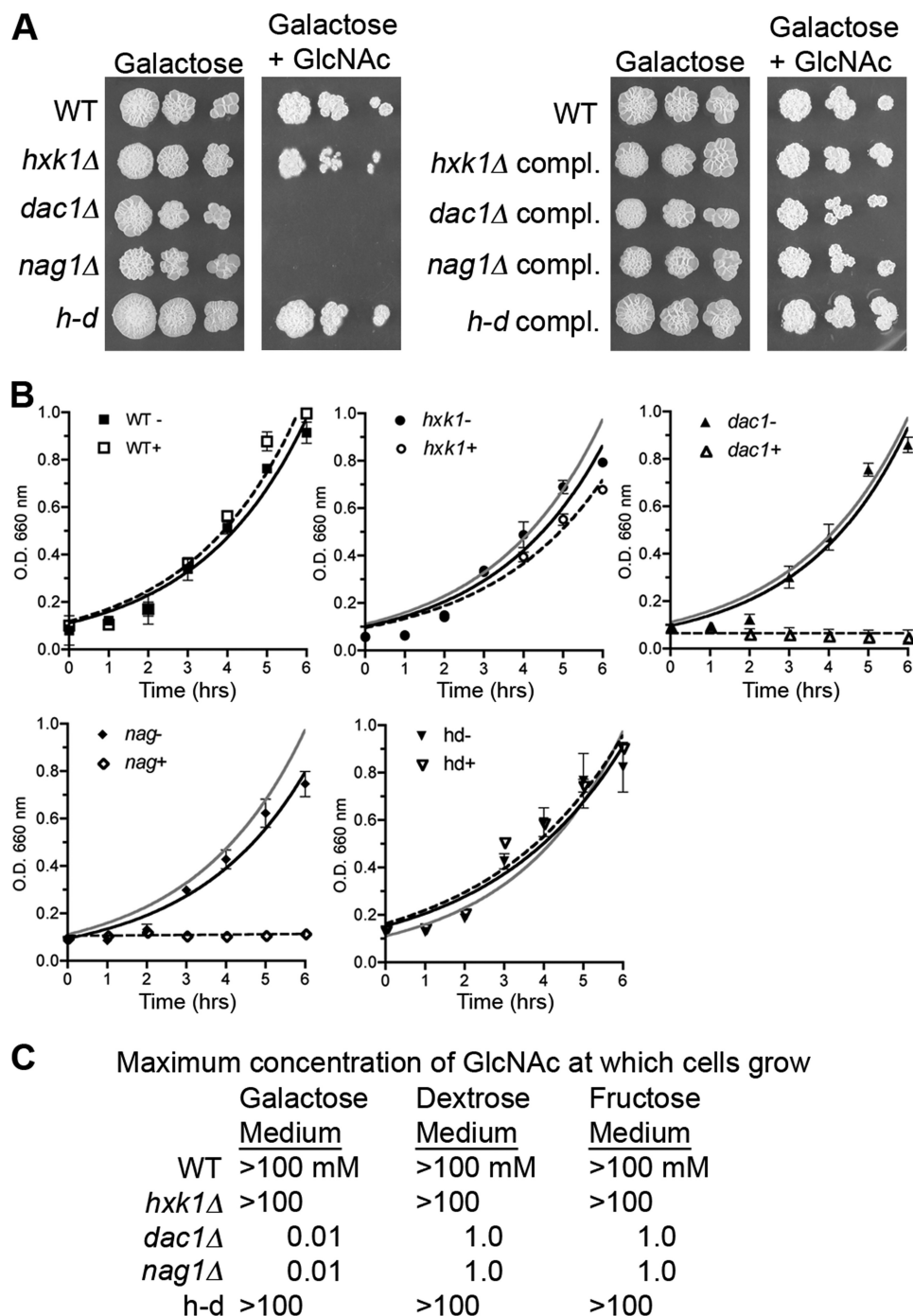


FIGURE 2. **GlcNAc inhibits growth of *nag1Δ* and *dac1Δ* mutants.** *A*, dilutions of the indicated strains were spotted onto medium containing 50 mM galactose plus or minus 10 mM GlcNAc and then incubated for 2 days at 37 °C. *B*, short term growth assays for cells incubated at 37 °C in medium containing 50 mM galactose plus (+) or minus (–) 10 mM GlcNAc as indicated. For comparison, the wild-type growth curve is shown as a gray line in the mutant growth curves. *C*, maximum concentration of GlcNAc at which cells can grow in synthetic medium that also contains 50 mM galactose, 50 mM dextrose, or 50 mM fructose. Cells were tested for growth in the presence of the indicated sugar plus 10-fold different concentrations of GlcNAc from 10 μ M to 100 mM. Strains are as described in the legend to Fig. 1. *compl.*, complemented.

induced efficiently, even at low doses of GlcNAc (e.g. 0.1 mM). Thus, GlcNAc catabolism is not needed for induction of hyphal growth.

A previous study reported that the *hxx1Δ*, *nag1Δ*, and *dac1Δ* mutants were defective in forming hyphae in response to serum (11). However, we found no defects in the ability of these mutants or the *h-d* mutant to be induced by serum (data not shown). Another study reported a defect in GlcNAc-induced hyphal for-

mation for a mutant in which *NAG1*, the promoter for *DAC1*, and a portion of *HXX1* were deleted (28). However, it appears that this mutant was primarily defective in forming new growth on GlcNAc medium, perhaps because of an inhibitory effect or the absence of a suitable carbon and energy source.

GlcNAc Catabolism Is Not Needed to Induce Expression of the NGT1, HXX1, NAG1, and DAC1 Genes—The ability of GlcNAc to activate the separate signaling pathway that induces expres-

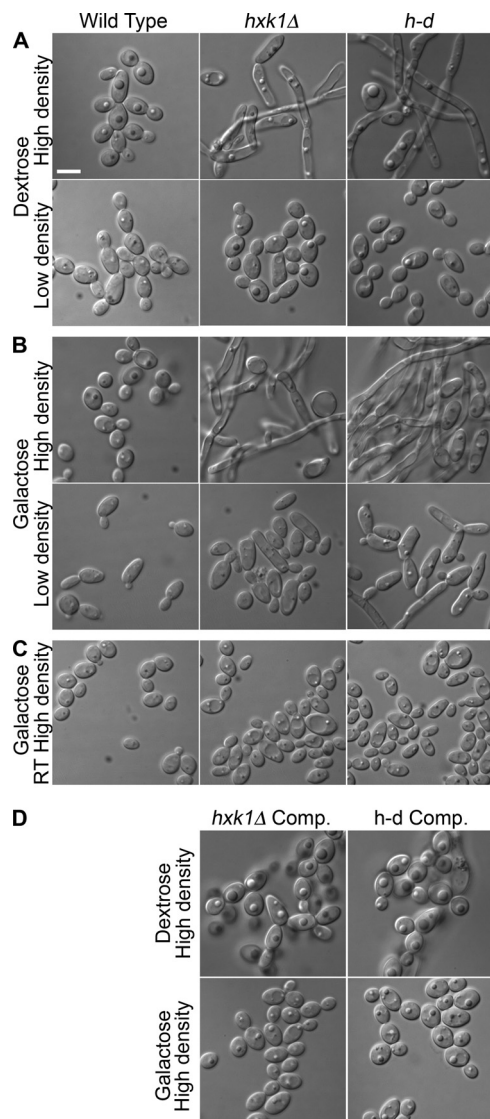


FIGURE 3. Mutant cell morphology varies with cell density in the absence of GlcNAc. Different dilutions of the wild-type control and mutant strains were grown overnight at 37 °C in synthetic medium containing either dextrose (A) or galactose (B). Light microscopic images were then captured for cells that were maintained in early log phase (labeled *Low density*, $\leq 10^6$ cells/ml) or grown to saturation (labeled *High density*, $\sim 10^8$ cells/ml). C, cells grown to high density in galactose medium at room temperature. D, complemented (*Comp.*) strains in which a copy of the wild-type gene was reintroduced into the mutant cells. Strains are as described in the legend to Fig. 1. Bar, 5 μ m.

sion of the catabolic genes was analyzed using qPCR. In dextrose medium, wild-type cells showed a low level of expression of the GlcNAc transporter *NGT1* that was induced about 23-fold when cells were switched to GlcNAc medium (Fig. 5A). The basal level of *NGT1* expression was higher in both the *hxk1Δ* and h-d mutants, analogous to what was observed for hyphal growth. *NGT1* was induced by GlcNAc in the *hxk1Δ* mutant to about the same level as the wild type, but because of the higher basal level the relative induction was 4.8-fold. Interestingly, despite the higher basal level of *NGT1* expression in the h-d mutant, GlcNAc still induced *NGT1* expression 15.4-fold. Thus, the overall GlcNAc-induced level of *NGT1* expression was about 8.8-fold higher in the h-d mutant than in the wild type. Similar results were observed for the expression of

the *NAG1* and *DAC1* genes in the *hxk1Δ* mutant. As expected, we failed to detect expression of *HXX1* in the *hxk1Δ* mutant or expression of the *HXX1*, *NAG1* or *DAC1* genes in the h-d mutant (Fig. 5A).

To determine whether the elevated *NGT1* expression observed in the h-d mutant was seen for other genes, we examined the expression of the GlcNAc-induced gene *GIG1*, which is thought to play a role in GlcNAc metabolism (8). Both the *hxk1Δ* and the h-d mutants showed higher basal levels of *GIG1* relative to the wild type (Fig. 5B). Although *GIG1* was poorly induced in the *hxk1Δ* mutant, it was highly induced in the h-d mutant. Similar to the relative levels of *NGT1* expression after GlcNAc induction, *GIG1* was expressed 7.9-fold higher in the h-d mutant than in the wild type. Additional control studies showed the expression of *PMA1*, the plasma membrane ATPase, was not significantly altered by GlcNAc induction in the h-d mutant (Fig. 5B). Thus, higher levels of GlcNAc-induced gene expression are seen in the h-d mutant.

Ngt1-GFP Is Induced by GlcNAc in the hxk1Δ and h-d Mutants—The results for GlcNAc-regulated gene expression were analyzed further by examining an *NGT1-GFP* reporter gene that was introduced into the wild-type and mutant cells. Control studies showed that wild-type cells grown in dextrose displayed no detectable *Ngt1-GFP* fluorescence, but *Ngt1-GFP* was detectable in the plasma membrane of cells grown in GlcNAc for 2 h (Fig. 6, A and B). Some GFP signal was also detected in the vacuole, presumably because of endocytosis. A high basal level of *Ngt1-GFP* was detected for a subset of the *hxk1Δ* and h-d mutant cells when grown in dextrose, consistent with the elevated expression of *NGT1* detected by qPCR. Interestingly, the fraction of cells producing detectable *Ngt1-GFP* increased with increasing cell density for the mutants but not for the wild type. Approximately 5% of the *nag1Δ* and *dac1Δ* mutants showed elevated levels of *Ngt1-GFP* when grown to a low cell density ($\sim 10^6$ cells/ml; Fig. 6A), but this increased to essentially 100% of the cells when they were grown to saturation ($\sim 10^8$ cells/ml; Fig. 6C). GlcNAc was able to highly induce *Ngt1-GFP* in the *hxk1Δ* and h-d mutants, similar to the induction of the *NGT1* mRNA detected by qPCR (Fig. 6B). Comparison of signal intensity in the digital images showed that *Ngt1-GFP* was induced to ~ 4 -fold higher in the *hxk1Δ* mutant and ~ 8 -fold higher in the h-d mutant relative to the level detected in the wild-type cells. Thus, the *Ngt1-GFP* results confirm the trends seen for the expression of GlcNAc-induced genes in the *hxk1Δ* and h-d mutants.

GlcNAc-induced Expression of Galactose-regulated Genes—Microarray analysis of gene expression in *C. albicans* unexpectedly revealed that GlcNAc stimulated the expression of a subset of galactose-regulated genes, *GAL1*, *GAL7*, and *GAL10* (8). GlcNAc catabolism is not needed for this induction, because qPCR analysis showed that GlcNAc induced *GAL10* to similar levels in the wild-type and *hxk1Δ* cells, and *GAL10* was hyperinduced in the h-d mutant (Fig. 7A). We previously speculated that the *GAL* genes are induced by activation of Cph1 in response to GlcNAc, because Cph1 is a transcription factor involved in inducing both hyphal genes and galactose-regulated genes (8, 29, 30). This prediction was also based on the fact that *GAL1*, -7, and -10 are the only genes in the *C. albicans* genome

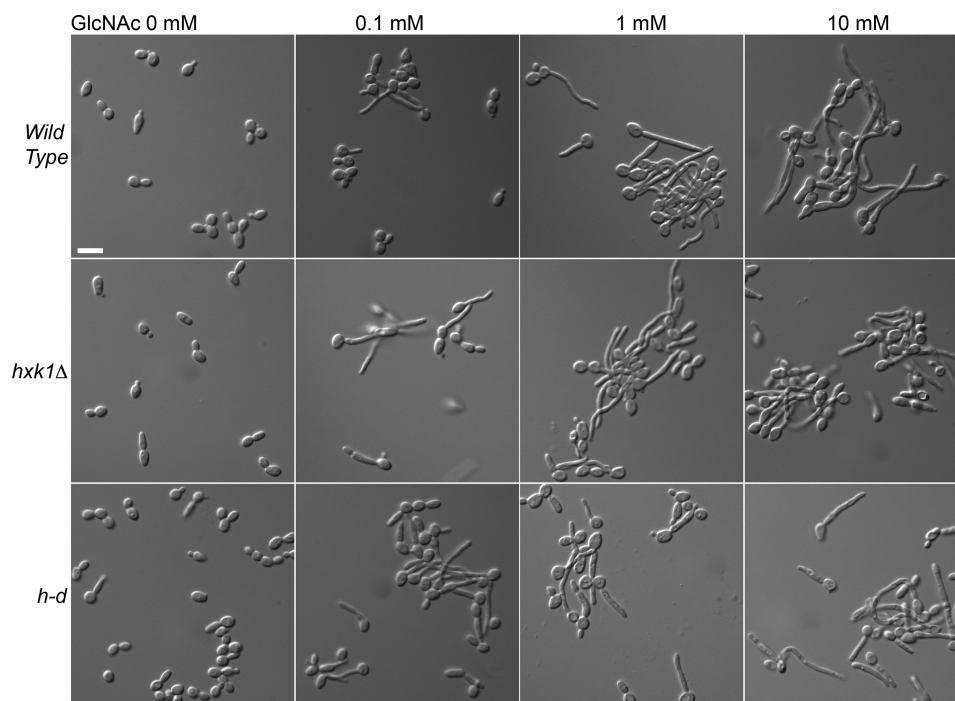


FIGURE 4. **GlcNAc induction of hyphal growth in the *hxx1* Δ and *h-d* mutants.** Cells were grown in synthetic medium containing 50 mM galactose plus the indicated concentration of GlcNAc for 2 h at 37 °C. The *hxx1* Δ and *h-d* strains were stimulated by GlcNAc at all concentrations tested to form elongated hyphae similar to the wild type. Strains used were the wild-type control (DIC185), *hxx1* Δ (AG736), and *h-d* (AG738) mutant. Bar, 10 μ m.

that contain typical Cph1-binding sites in their promoters (29). To test this idea, we examined the ability of GlcNAc to induce gene expression in two independently constructed Cph1 mutants (31, 32), both of which were confirmed to lack *CPH1* by PCR methods. Surprisingly, GlcNAc efficiently induced *GAL10* in the *cph1* Δ mutants (Fig. 7B). Thus, these results demonstrate that the induction of *GAL10* does not require GlcNAc stimulation of Cph1. They also demonstrate that Cph1 is not needed to induce *NGT1*, consistent with the ability of *cph1* Δ mutant cells to grow well on GlcNAc medium (see below).

Mkc1 and Cek1 Are Not Needed for GlcNAc Signaling—The Mkc1 and Cek1 MAPKs regulate cell-signaling pathways in *C. albicans* and were therefore tested for a role in GlcNAc signaling. However, mutant cells lacking these kinases grew well on GlcNAc medium and could be induced to form hyphae in response to GlcNAc (Fig. 8). A *cph1* Δ mutant also grew well on GlcNAc, consistent with its ability to induce GlcNAc catabolic genes (Fig. 7). However, as expected from previous studies (33), this mutant displayed a partial defect in forming hyphae in response to both GlcNAc and serum. An *efg1* mutant that lacks a transcription factor activated by cAMP signaling showed a stronger defect in hyphal induction, as expected from previous studies (33), but it still grew well on GlcNAc medium. Thus, these genes do not play an essential role in GlcNAc signaling.

DISCUSSION

Nonphosphorylated GlcNAc Activates Cell Signaling in *C. albicans*—GlcNAc plays important roles in cell structure and signaling in a wide range of organisms from bacteria to man. However, the mechanisms of GlcNAc signaling are not well understood. Previous studies of the human fungal pathogen *C. albicans* indicated that GlcNAc must be transported into the

cells to stimulate hyphal morphogenesis and gene expression (12). Therefore, mutant cells lacking the GlcNAc catabolic genes were analyzed to determine whether GlcNAc or a subsequent breakdown product was important for signaling. Interestingly, *hxx1* Δ cells lacking the GlcNAc kinase or the *h-d* mutant that lacks all three catabolic genes (*hxx1* Δ *nag1* Δ *dac1* Δ) could be induced efficiently to undergo hyphal morphogenesis (Fig. 4) and turn on expression of GlcNAc-regulated genes (Fig. 5). These results indicate that GlcNAc does not have to be phosphorylated and catabolized to activate signaling. The phenotypes of the *hxx1* Δ and *h-d* mutants also indicate that GlcNAc does not have to enter the anabolic pathways to induce signaling. Phosphorylation of exogenous GlcNAc by Hxk1 is required to create GlcNAc-6-PO₄, which is then converted to UDP-GlcNAc for use as a substrate in the anabolic pathways that form chitin, *N*-linked glycosylation, and glycosylphosphatidylinositol anchors on proteins (16). Thus, the results indicate that GlcNAc metabolism is not required for it to activate signaling.

The discovery that nonphosphorylated GlcNAc from exogenous sources can activate signaling in *C. albicans* therefore identifies a novel role for GlcNAc in cell signaling that may be used to regulate intercellular signaling in other organisms, including humans. GlcNAc signaling in *C. albicans* does not require *O*-GlcNAc transferase as in humans, because this enzyme uses UDP-GlcNAc as a substrate for protein modification (5). This conclusion is also supported by the lack of a detectable *O*-GlcNAc transferase gene in *C. albicans*. Sensing nonphosphorylated GlcNAc would also have the advantage of allowing cells to distinguish exogenous nonphosphorylated GlcNAc that is transported into the cell from the

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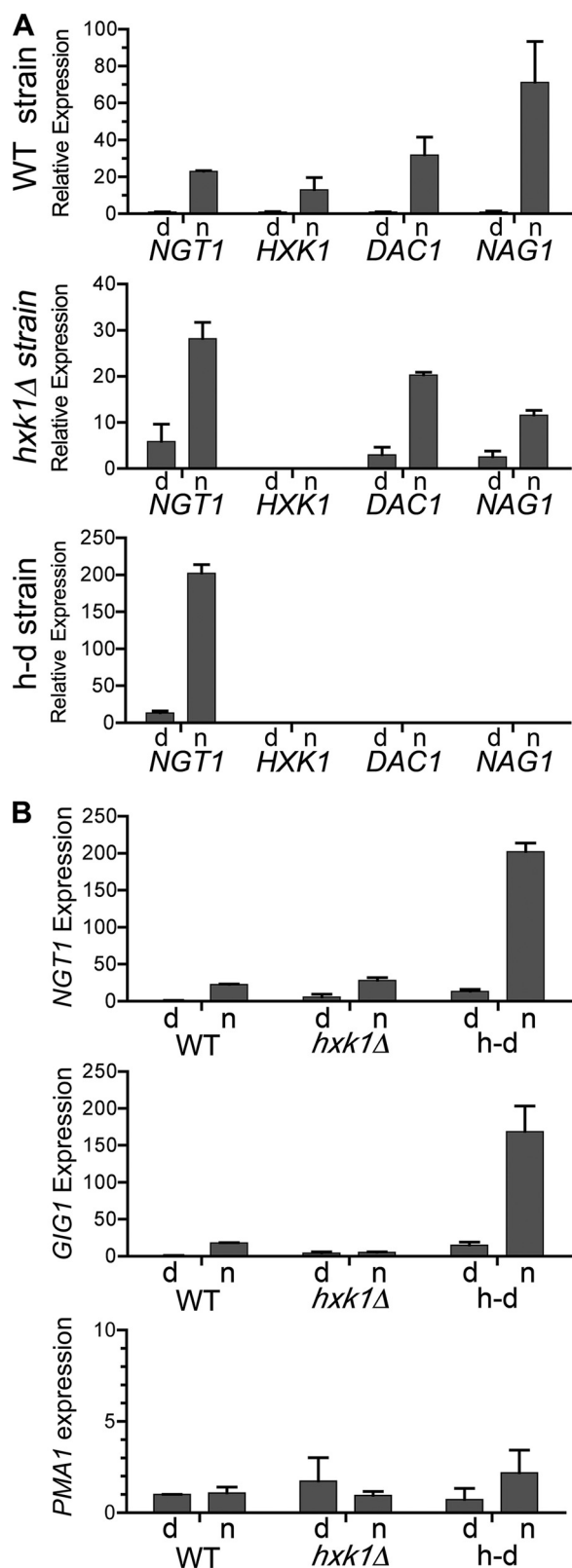


FIGURE 5. GlcNAc induces the catabolic genes in the mutant cells. A, expression of the GlcNAc transporter (*NGT1*), GlcNAc-6-PO₄ deacetylase (*DAC1*), and glucosamine-6-PO₄ deaminase (*NAG1*) genes. The graphs for the wild-type control, *hdk1Δ*, and h-d strains are indicated on the left. Cells were grown in medium containing 50 mM dextrose (d) or 50 mM *N*-acetylglucosamine (n) for 2 h at 37 °C. B, relative expression of *NGT1*, the GlcNAc-induced gene *GIG1*, and *PMA1*. The level of expression for each gene was normalized to that for the wild-type control strain grown in dextrose. Note that *PMA1* is a control gene that is not regulated by GlcNAc. The mRNA levels were analyzed

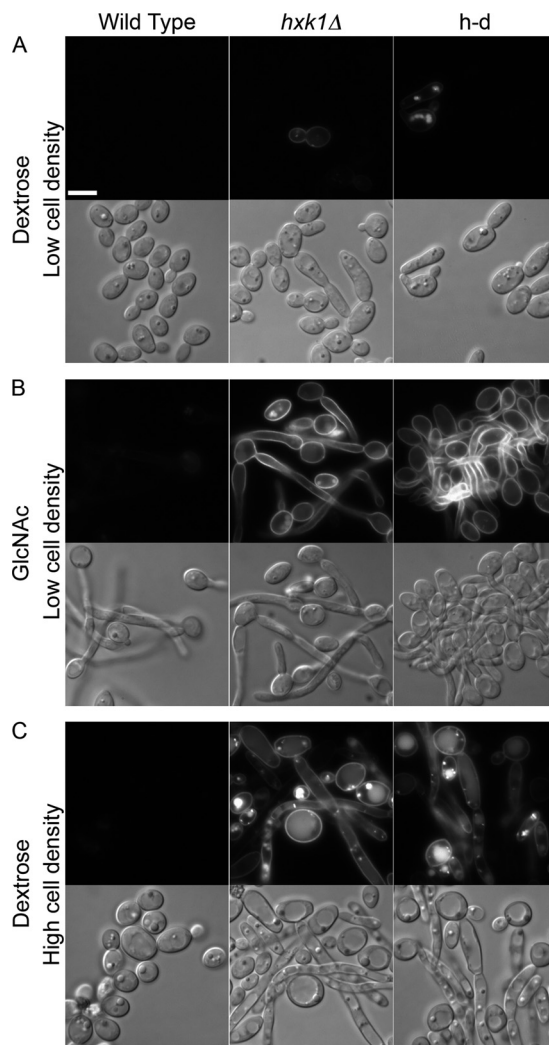


FIGURE 6. GlcNAc induction of Ngp1-GFP in *hdk1Δ* and h-d mutants. Analysis of Ngp1-GFP production in cells grown overnight in dextrose medium to early log phase (low cell density) and then switched to medium containing 50 mM dextrose (A) or 50 mM GlcNAc (B) for 2 h. C, cells grown overnight to saturation (High cell density) in medium containing 50 mM dextrose. The cells were then photographed using a fluorescence microscope to detect GFP. A gene fusion between the GlcNAc transporter and the green fluorescent protein (*NGT1-GFP*) was introduced into cells to create a wild-type control strain (SN787), an *hdk1Δ* mutant (SN791), and an h-d mutant (SN794). Bar, 5 μm.

GlcNAc-6-PO₄ that is synthesized within the cell. Intracellular GlcNAc synthesis involves conversion of fructose-6-PO₄ to glucosamine-6-PO₄ and then to GlcNAc-6-PO₄ (16). Detecting nonphosphorylated GlcNAc is also expected to provide increased sensitivity for sensing extracellular GlcNAc, because there is a high rate of flux of GlcNAc-6-PO₄ through the anabolic pathways in cells to keep up with the demands for the structural roles of GlcNAc. The ability of *C. albicans* to sense nonphosphorylated GlcNAc is also supported by the observation that glucosamine does not induce hyphal morphogenesis. Glucosamine is expected to be phosphorylated to glucosamine-

by qPCR as described under "Experimental Procedures." The results represent the mean ± S.D. of four independent assays each carried out in triplicate. The data include analyses of two completely independent mutant isolates and RNA preparations for each strain.

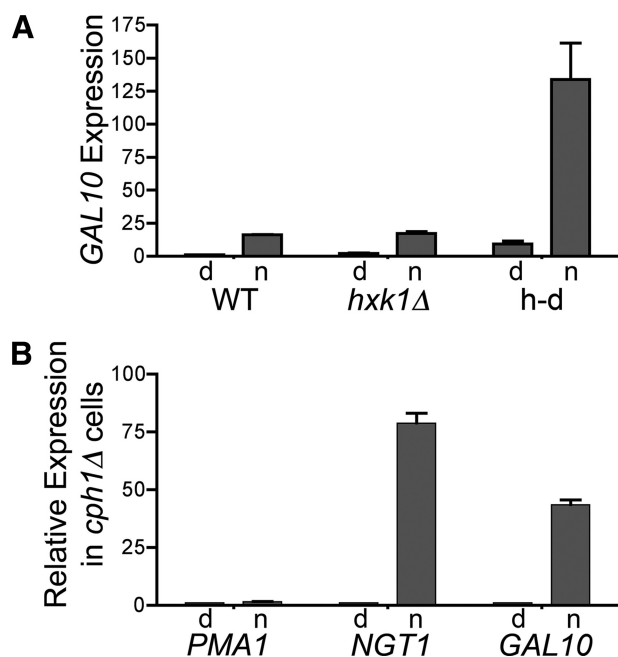


FIGURE 7. **GlcNAc induction of *GAL10* is independent of *CPH1*.** *A*, relative *GAL10* mRNA levels were analyzed by qPCR for the wild-type strain (DIC185), *hxx1Δ* (AG736), and h-d (AG738). *B*, *PMA1*, *NGT1*, and *GAL10* expression levels in *cph1Δ* mutant cells. The data represent the mean \pm S.D. for RNA preparations obtained from two different *cph1Δ* strains (strains 180 and CJN305). Cells were grown in synthetic medium containing dextrose (d) or N-acetylglucosamine (n), and then qPCR was performed as described under "Experimental Procedures."

6-PO₄ and then converted to GlcNAc-6-PO₄ or catabolized to fructose-6-PO₄ (16).

Other sugar signaling pathways also sense nonphosphorylated sugars. For example, *S. cerevisiae* Gal3, an enzymatically inactive paralog of the galactose kinase Gal1, binds nonphosphorylated galactose and then migrates to the nucleus to stimulate Gal4 to induce gene expression by removing the inhibitory Gal80 factor (13). Similarly, the *S. cerevisiae* glucose kinase Hxk2 binds glucose and then effects changes in transcription in the nucleus (34). Although these sugar kinases have been directly implicated in gene regulation stimulated by other carbohydrates, analysis of the *hxx1Δ* and h-d mutants indicates that the GlcNAc kinase Hxk1 is not needed for induction of GlcNAc-regulated genes in *C. albicans* (Fig. 5).

Inhibitory Effects of GlcNAc on *nag1Δ* and *dac1Δ* Mutants—The *nag1Δ* and *dac1Δ* mutants quickly ceased growth in the presence of GlcNAc, even when another sugar was available for nutrition (Fig. 2). The inability of these mutants to deacetylate and deaminate GlcNAc-6-PO₄ suggests that the resulting excess of GlcNAc-6-PO₄ is toxic to cells. Similar deleterious effects that are attributable to excess GlcNAc-6-PO₄ have been observed in other mutant organisms. For example, GlcNAc inhibited growth of *S. cerevisiae* cells that express *C. albicans* *HXX1* but not cells that co-expressed *HXX1* with *NAG1* and *DAC1* (15). GlcNAc also inhibited the growth of mutant *E. coli* cells that lack GlcNAc deacetylase or deaminase activity (25). Growth was quickly restored by adding exogenous uridine (26), suggesting that conversion of excess GlcNAc-6-PO₄ to UDP-GlcNAc may have depleted uridine levels. In contrast, extra uridine did not quickly rescue the *C. albicans* *nag1Δ* and

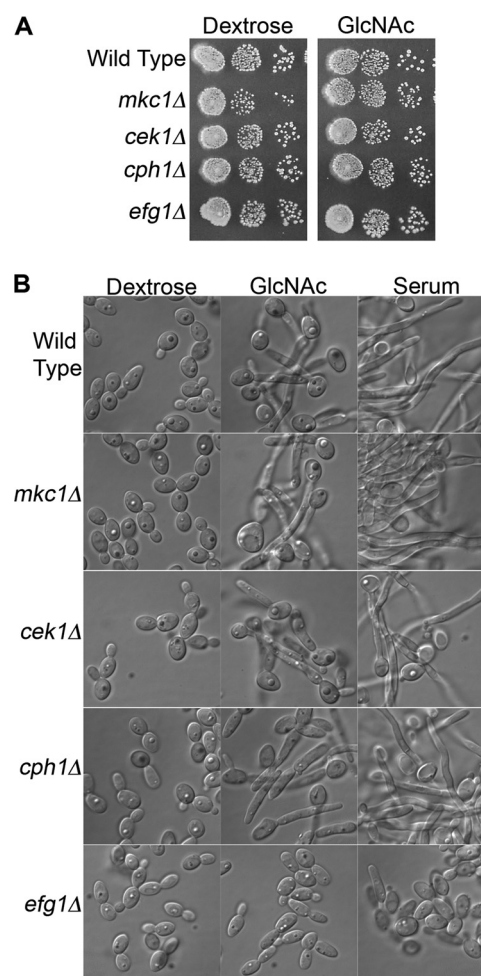


FIGURE 8. ***Mck1*, *Cek1*, *Cph1*, and *Efg1* are not required for GlcNAc catabolism or GlcNAc induction of hyphal growth.** *A*, dilutions of a wild-type control and the indicated mutant strains were spotted onto solid agar media plates containing 50 mM of the indicated sugar and then incubated at 37 °C. *B*, cells were grown overnight to log phase and then incubated in medium containing 50 mM dextrose, 50 mM GlcNAc, or 50 mM dextrose plus 10% serum at 37 °C for 2 h. The wild-type control (SN250), *mck1* (1112), *cek1* (544), and *cph1Δ* (180) strains were described by Noble *et al.* (32), and the *efg1* strain (060) was described by Homann *et al.* (40).

dac1Δ mutants from inhibition by GlcNAc (Fig. 2). Another example of this type of inhibition was reported for *Leishmania*, in which the GlcNAc sensitivity of a *gnd* mutant lacking glucosamine deaminase was attributed to depletion of ATP (35). The inhibitory effect of GlcNAc on the *Leishmania gnd* mutant appears to be distinct from that seen for the *C. albicans* mutants because it could be overcome by adding alternative carbon sources, such as glycerol.

It is interesting to speculate that the inhibitory effects of GlcNAc on *nag1Δ* and *dac1Δ* mutants could have played a role in loss of the GlcNAc catabolic genes in *S. cerevisiae*, *S. pombe*, and related species. The *HXX1*, *NAG1*, and *DAC1* genes are in a cluster termed the NAG regulon in *C. albicans* that is also conserved in other species (36). This clustering of genes might facilitate their simultaneous loss in the case of an adverse mutation of *NAG1* or *DAC1*.

Activation of *GAL* Genes—GlcNAc induction of a subset of the galactose-regulated genes was unexpected, because there is no obvious overlap between galactose and GlcNAc catabolism

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(8). Furthermore, GlcNAc catabolism was not required for *GAL10* to be induced in the *hvk1Δ* and *h-d* mutants (Fig. 7), and galactose does not induce GlcNAc catabolic genes (29, 37). We previously proposed that GlcNAc induction of a subset of *GAL* genes might be due to activation of Cph1 (8), a transcription factor that is implicated in the regulation of both hyphal- and galactose-stimulated genes (29, 30). The *GAL1*, *GAL7*, and *GAL10* genes that were induced by GlcNAc were reported to be the only *C. albicans* genes that contain a putative Cph1 recognition motif in their upstream regulatory regions (29). Other galactose-stimulated genes whose promoters lack this recognition site were not induced by GlcNAc (8, 29). Despite this suggestive evidence, two independently constructed *cph1Δ* mutants could be stimulated by GlcNAc to induce *GAL10* efficiently (Fig. 7), demonstrating that Cph1 is not required for this effect. Nonetheless, the induction of *GAL1*, *GAL7*, and *GAL10* by GlcNAc may be physiologically significant as it has been suggested that *GAL10* functions in cell wall integrity even in the absence of galactose (38).

GlcNAc and Virulence—Previous studies observed that *C. albicans* *hvk1Δ*, *dac1Δ*, and *nag1Δ* mutants were defective for pathogenesis in a mouse model of systemic infection and concluded that GlcNAc catabolism was important for virulence (11, 28). However, the relationship between GlcNAc catabolism and virulence is unclear. One reason is that, unfortunately, the gene deletion methods used in these studies are now known to require additional controls before they can be reliably used to assess virulence (23, 24). Furthermore, the new phenotypes uncovered for the GlcNAc catabolic mutants could affect pathogenesis independently of the ability to use GlcNAc as a nutrient source. For example, the *hvk1Δ* and *h-d* mutants display a higher basal level of filamentous growth (Fig. 3) that could decrease virulence similar to the *C. albicans* mutants that are locked into the hyphal mode of growth (39). Also, the strong inhibitory effects of GlcNAc on the proliferation of the *nag1Δ* and *dac1Δ* mutants could impact growth *in vivo* in some niches (Fig. 2). It is unclear whether *C. albicans* cells in a systemic infection typically encounter much GlcNAc, because serum is low in GlcNAc and high in glucose. However, GlcNAc catabolism is likely to be important for commensal growth of *C. albicans* on mucosal surfaces. The extracellular matrix glycosaminoglycans that line the mucosa are rich in GlcNAc. GlcNAc is also expected to be present in the gastrointestinal tract because of remodeling of the bacterial peptidoglycan. Thus, GlcNAc plays a multifaceted role in cell signaling and structure of *C. albicans* during commensal growth and pathogenesis.

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REFERENCES

1. Barnhart, M. M., Lynem, J., and Chapman, M. R. (2006) *J. Bacteriol.* **188**, 5212–5219
2. Simonetti, N., Strippoli, V., and Cassone, A. (1974) *Nature* **250**, 344–346
3. Pérez-Campo, F. M., and Domínguez, A. (2001) *Curr. Microbiol.* **43**, 429–433
4. Hart, G. W., Housley, M. P., and Slawson, C. (2007) *Nature* **446**, 1017–1022
5. Slawson, C., Copeland, R. J., and Hart, G. W. (2010) *Trends Biochem. Sci.* **35**, 547–555
6. Castilla, R., Passeron, S., and Cantore, M. L. (1998) *Cell. Signal.* **10**, 713–719
7. Leberer, E., Harcus, D., Dignard, D., Johnson, L., Ushinsky, S., Thomas, D. Y., and Schröppel, K. (2001) *Mol. Microbiol.* **42**, 673–687
8. Gunasekera, A., Alvarez, F. J., Douglas, L. M., Wang, H. X., Rosebrock, A. P., and Konopka, J. B. (2010) *Eukaryot. Cell* **9**, 1476–1483
9. Huang, G., Yi, S., Sahni, N., Daniels, K. J., Srikantha, T., and Soll, D. R. (2010) *PLoS Pathog.* **6**, e1000806
10. Kumar, M. J., Jamaluddin, M. S., Natarajan, K., Kaur, D., and Datta, A. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97**, 14218–14223
11. Yamada-Okabe, T., Sakamori, Y., Mio, T., and Yamada-Okabe, H. (2001) *Eur. J. Biochem.* **268**, 2498–2505
12. Alvarez, F. J., and Konopka, J. B. (2007) *Mol. Biol. Cell* **18**, 965–975
13. Sellick, C. A., and Reece, R. J. (2005) *Trends Biochem. Sci.* **30**, 405–412
14. Douglas, H. C., and Condie, F. (1954) *J. Bacteriol.* **68**, 662–670
15. Wendland, J., Schaub, Y., and Walther, A. (2009) *Appl. Environ. Microbiol.* **75**, 5840–5845
16. Milewski, S., Gabriel, I., and Olchoway, J. (2006) *Yeast* **23**, 1–14
17. Sherman, F. (1991) *Methods Enzymol.* **194**, 3–21
18. Wilson, R. B., Davis, D., and Mitchell, A. P. (1999) *J. Bacteriol.* **181**, 1868–1874
19. Wilson, R. B., Davis, D., Enloe, B. M., and Mitchell, A. P. (2000) *Yeast* **16**, 65–70
20. Park, H., Myers, C. L., Sheppard, D. C., Phan, Q. T., Sanchez, A. A., Edwards, J., and Filler, S. G. (2005) *Cell. Microbiol.* **7**, 499–510
21. Zhang, C., and Konopka, J. B. (2010) *Eukaryot. Cell* **9**, 224–226
22. Natarajan, K., and Datta, A. (1993) *J. Biol. Chem.* **268**, 9206–92014
23. Lay, J., Henry, L. K., Clifford, J., Koltin, Y., Bulawa, C. E., and Becker, J. M. (1998) *Infect. Immun.* **66**, 5301–5306
24. Chen, X., Magee, B. B., Dawson, D., Magee, P. T., and Kumamoto, C. A. (2004) *Mol. Microbiol.* **51**, 551–565
25. White, R. J. (1968) *Biochem. J.* **106**, 847–858
26. Bernheim, N. J., and Dobrogosz, W. J. (1970) *J. Bacteriol.* **101**, 384–391
27. Wendland, J., Hellwig, D., Walther, A., Sickinger, S., Shadkhan, Y., Martin, R., Bauer, J., Oshero, N., Tretiakov, A., and Saluz, H. P. (2006) *J. Basic Microbiol.* **46**, 513–523
28. Singh, P., Ghosh, S., and Datta, A. (2001) *Infect. Immun.* **69**, 7898–7903
29. Martchenko, M., Levitin, A., Hogues, H., Nantel, A., and Whiteway, M. (2007) *Curr. Biol.* **17**, 1007–1013
30. Liu, H., Köhler, J., and Fink, G. R. (1994) *Science* **266**, 1723–1726
31. Nobile, C. J., and Mitchell, A. P. (2005) *Curr. Biol.* **15**, 1150–1155
32. Noble, S. M., French, S., Kohn, L. A., Chen, V., and Johnson, A. D. (2010) *Nat. Genet.* **42**, 590–598
33. Lo, H. J., Köhler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G. R. (1997) *Cell* **90**, 939–949
34. Gancedo, J. M. (2008) *FEMS Microbiol. Rev.* **32**, 673–704
35. Naderer, T., Heng, J., and McConville, M. J. (2010) *PLoS Pathog.* **6**, e1001245
36. Fitzpatrick, D. A., O'Gaora, P., Byrne, K. P., and Butler, G. (2010) *BMC Genomics* **11**, 290
37. Brown, V., Sabina, J., and Johnston, M. (2009) *Curr. Biol.* **19**, 436–441
38. Singh, V., Satheesh, S. V., Raghavendra, M. L., and Sadhale, P. P. (2007) *Fungal. Genet. Biol.* **44**, 563–574
39. Whiteway, M., and Oberholzer, U. (2004) *Curr. Opin. Microbiol.* **7**, 350–357
40. Homann, O. R., Dea, J., Noble, S. M., and Johnson, A. D. (2009) *PLoS Genet.* **5**, e1000783