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 morphology, 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 (HXX1, 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 hxx1Δ and an hxx1Δ 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 Hxx1 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-
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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 *dac1*Δ/*dac1*Δ and *nag1*Δ/*nag1*Δ deletion mutants that removed the entire open reading frames were constructed in *C. albicans* strain BWP17 (*arg4*Δ *his1*Δ *ura3*Δ) 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 *nag1*Δ and *dac1*Δ 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 pDD957 (19). The resulting *DAC1* plasmid was linearized in the promoter region by digestion with PstI and integrated into the *dac1*Δ/*dac1*Δ 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 *nag1*Δ/*nag1*Δ strain to create the complemented strain AG767. To create prototrophic versions of the *dac1*Δ/*dac1*Δ and *nag1*Δ/*nag1*Δ mutants, they were transformed with a *URA3*-containing DNA fragment to restore *URA3* at its native locus. The *URA3* frag-
ments were liberated from plasmid pBSK-URA3 (20) by digestion with restriction enzymes PstI and NotI.

For reasons that are unclear, homozygous deletions 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 hxk1Δ mutant AG736 and the h-d mutant (hxk1Δ nag1Δ dac1Δ) AG738. Complementing plasmids were then created using a derivative of pHDB57 in which URA3 was replaced with HIS1. The complementing plasmids were linearized by digestion with SplI 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 (CaGFPγ) 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. Fluorescence 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 × 10^8 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 (ΔΔC_{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-AGAAGCTTGGTTCCAGACCCGC-3' and ACT1-R 5'-TGTAGCTTCGAATATCCCTGGG-3'; NGT1-F 5'-TGGTGGCCAA-AATTGGTTGGGCT-3' and NGT1-R 5'-TGGGACATGGGTCCTCCAATACCCCA-3'; HXK1-F 5'-TGTGAGCTCTCGTTGTTTGG-3' and HXK1-R 5'-TCAATTTCCGCGATACTTCC-3'; NAG1-F 5'-GAAGCCGGATCATCAAGAAA-3' and NAG1-R 5'-TGGCAATTTCTGTCTGATTG-3'; DAC1-F 5'-GGTTGGCGCAATGTTGGCT-3' and DAC1-R 5'-GACGACTTGGACTTCAGCCTCCA-3'; GIG1-F 5'-GCAAACCACCCAGACCTTCACCA-3' and GIG1-R 5'-TGTTTGCTGTGATGACGACGCA-3'; GAL10-F 5'-AGAGCAGAAAACATTGCATGGTG-3' and GAL10-R 5'-GCTTCAGCTCACCTGGAGGAC-3'; and PMA1-F 5'-TGCCGAAATTGTGGGGTTGAT-3' and PMA1-R 5'-GACAGGAATGGACCTTGAGC-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 hxk1Δ, 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 hxk1Δ 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 for 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

4The 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), hxxk1Δ (AG733), dac1Δ (AG732), nag1Δ (AG734), and a triple hxxk1Δ 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 hxxk1Δ + 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 hxxk1Δ mutant but no effect on the growth of the h-d mutant. The observation that the hxxk1Δ and h-d mutants grow well in the presence of GlCNAC indicates that it must be converted to GlCNAC-6-P-O4 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-
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 \( \text{hxk1}\), \( \text{nag1}\), and \( \text{dac1}\) 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 formation for a mutant in which \( \text{NAG1} \), the promoter for \( \text{DAC1} \), and a portion of \( \text{HXK1} \) 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 \( \text{NGT1}, \text{HXK1}, \text{NAG1}, \text{and DAC1} \) Genes**

The ability of GlcNAc to activate the separate signaling pathway that induces expres-
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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, ~10^6 cells/ml) or grown to saturation (labeled high density, ~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 μm.

Expression 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 hxxk1Δ and h-d mutants, analogous to what was observed for hyphal growth. NGT1 was induced by GlcNAc in the hxxk1Δ 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 hxxk1Δ mutant. As expected, we failed to detect expression of HXK1 in the hxxk1Δ mutant or expression of the HXK1, 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 hxxk1Δ 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 hxxk1Δ 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 hxxk1Δ 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 hxxk1Δ 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 (~10^6 cells/ml; Fig. 6A), but this increased to essentially 100% of the cells when they were grown to saturation (~10^8 cells/ml; Fig. 6C). GlcNAc was able to highly induce Ngt1-GFP in the hxxk1Δ 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 hxxk1Δ 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 hxxk1Δ 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 hxxk1Δ cells, and GAL10 was hyper-induced 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

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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/H9004 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/H9004 mutant cells to grow well on GlcNAc medium (see below).

**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, hsk1Δ cells lacking the GlcNAc kinase or the h-d mutant that lacks all three catabolic genes (hsk1Δ nag1Δ dac1Δ) 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 hsk1Δ 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
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 anaerobic 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.

**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, *hxk1Δ*, 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 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.
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 *hxk1*/*H9004* 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 *nag1*/*H9004* and *dac1*/*H9004* Mutants—**
The *nag1*Δ and *dac1*Δ 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* HXK1 but not cells that co-expressed HXK1 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* *nag1*Δ and *dac1*Δ 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 *nag1*Δ and *dac1*Δ mutants could have played a role in loss of the GlcNAc catabolic genes in *S. cerevisiae*, *S. pombe*, and related species. The HXK1, 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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**FIGURE 7.** GlcNAc induction of GAL10 is independent of CP1H1. A, relative GAL10 mRNA levels were analyzed by qPCR for the wild-type strain (DIC185), *hxk1*Δ (AG736), and h-d (AG738). B, PMA1, NGT1, and GAL10 expression levels in cph1Δ mutant cells. The data represent the mean ± 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.”

**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 mutants 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), *mkc1* (1112), *cek1* (544), and *cph1*Δ (180) strains were described by Noble et al. (32), and the *efg1* strain (060) was described by Homann et al. (40).
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(8). Furthermore, GlcNAc catabolism was not required for GAL10 to be induced in the hxx1Δ 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 hxx1Δ, 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 hxx1Δ and h-d mutants display a higher basal level of filamentous growth (Fig. 3) that could decrease virulence similar to the cph1Δ 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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