Identification of an *N*-Acetylglucosamine Transporter That Mediates Hyphal Induction in *Candida albicans*[□]

Francisco J. Alvarez* and James B. Konopka[†]

*Graduate Program in Genetics and [†]Department of Molecular Genetics and Microbiology, State University of New York, Stony Brook, NY 11794-5222

Submitted October 19, 2006; Revised December 5, 2006; Accepted December 20, 2006
Monitoring Editor: Daniel Lew

The sugar *N*-acetylglucosamine (GlcNAc) plays an important role in nutrient sensing and cellular regulation in a wide range of organisms from bacteria to humans. In the fungal pathogen *Candida albicans*, GlcNAc induces a morphological transition from budding to hyphal growth. Proteomic comparison of plasma membrane proteins from buds and from hyphae induced by GlcNAc identified a novel hyphal protein (Ngt1) with similarity to the major facilitator superfamily of transporters. An Ngt1-GFP fusion was detected in the plasma membrane after induction with GlcNAc, but not other related sugars. Ngt1-GFP was also induced by macrophage phagocytosis, suggesting a role for the GlcNAc response in signaling entry into phagolysosomes. *NGT1* is needed for efficient GlcNAc uptake and for the ability to induce hyphae at low GlcNAc concentrations. High concentrations of GlcNAc could bypass the need for *NGT1* to induce hyphae, indicating that elevated intracellular levels of GlcNAc induce hyphal formation. Expression of *NGT1* in *Saccharomyces cerevisiae* promoted GlcNAc uptake, indicating that Ngt1 acts directly as a GlcNAc transporter. Transport mediated by Ngt1 was specific, as other sugars could not compete for the uptake of GlcNAc. Thus, Ngt1 represents the first eukaryotic GlcNAc transporter to be discovered. The presence of *NGT1* homologues in the genome sequences of a wide range of eukaryotes from yeast to mammals suggests that they may also function in the cellular processes regulated by GlcNAc, including those that underlie important diseases such as cancer and diabetes.

INTRODUCTION

N-acetylglucosamine (GlcNAc) is an amino sugar that carries out important roles in a broad range of cells from bacteria to humans. One aspect of GlcNAc function is to mediate cellular signaling. In bacteria, GlcNAc induces components that are important for colonization of human hosts, including fimbrins that mediate adhesion to host cells (Sohanpal *et al.*, 2004), multidrug exporter genes (Hirakawa *et al.*, 2006) and Curli fibers that promote biofilm formation (Barnhart *et al.*, 2006). In mammals, GlcNAc is a key sensor of nutrient status that is involved in insulin signaling, cell cycle control, and other essential processes. Nutritional effects that lead to increased GlcNAc levels result in its conversion to UDP-GlcNAc and subsequent attachment to proteins on serine or threonine residues in a dynamic manner that is analogous to modification of proteins by phosphorylation (Slawson and Hart, 2003; Zachara and Hart, 2006). In fact, the interplay between *O*-GlcNAc modification and phosphorylation of proteins regulates many critical transcription factors, such as *c-myc* and *p53* (Chou and Hart, 2001; Yang *et al.*, 2006). *O*-GlcNAc modification also regulates other processes including proteasome function (Zachara and Hart, 2004). In addition to these regulatory roles, GlcNAc contributes to the *N*-linked glycosylation, glycosylphosphatidylinositol (GPI)

anchor addition to proteins and is polymerized into chitin, which forms part of the fungal cell wall and the exoskeletons of parasites, insects, and other organisms.

GlcNAc induces the fungal pathogen *Candida albicans* to switch from forming round budding cells to instead growing as long thin hyphal cells. This morphological transition is thought to be an important virulence factor (Sudbery *et al.*, 2004; Whiteway and Oberholzer, 2004). *C. albicans* is one of the leading causes of nosocomial bloodstream infections and is capable of causing severe systemic infections (Edmond *et al.*, 1999; Mavor *et al.*, 2005; Spellberg *et al.*, 2006). *C. albicans* can be detected at sites of infection in various morphologies including rounded buds, pseudohyphae (chains of elongated buds), and hyphae (chains of long thin cells with parallel walls) (Odds, 1988). Each form is thought to confer a distinct advantage during infection. For example, small buds are more likely to disseminate in the bloodstream and hyphal cells may be better suited for invasive growth into tissues. The distinct cell types also differ in their production of virulence factors, such as the adhesin proteins that mediate attachment to host cells and secreted hydrolytic enzymes that facilitate invasive growth (Whiteway and Oberholzer, 2004; Kumamoto and Vines, 2005).

In vitro studies have identified a variety of different stimuli that can induce *C. albicans* to switch morphologies. One of the strongest known inducers of hyphal growth is serum. Screens for chemically defined inducers have found that nutrients, such as the amino sugar GlcNAc (Simonetti *et al.*, 1974) and certain amino acids (Odds, 1988; Maidan *et al.*, 2005), will also stimulate hyphal growth. Environmental conditions also contribute to hyphal induction, as it occurs optimally at 37°C and can be stimulated by alkaline pH and 5% CO₂ (Davis, 2003; Sudbery *et al.*, 2004; Klengel *et al.*, 2005). The upstream components of the hyphal signal path-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-10-0931>) on December 27, 2006.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: James B. Konopka (james.konopka@sunysb.edu).

Table 1. Strains used in this study

Strain	Parent	Genotype
<i>C. albicans</i> strains		
Sc5314		Wild type
BWP17	Sc5314	<i>ura3Δ::λimm434/ura3Δ::λimm434 his::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
DIC185	BWP17	<i>ura3Δ::λimm434/URA3 his::hisG/HIS1 arg4::hisG/ARG4</i>
YJA1	CAI4	<i>ura3Δ::imm434/ura3Δ::imm434 NGT1-GFP-URA3/NGT1</i>
YJA2	BWP17	<i>ngt1Δ::ARG4/ngt1Δ::HIS1 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
YJA3	YJA2	<i>ngt1Δ::ARG4/ngt1Δ::HIS1 ura3Δ::λimm434/URA3 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
YJA4	YJA2	<i>ngt1Δ::ARG4/ngt1Δ::HIS1::NGT1-URA3 ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>
<i>S. cerevisiae</i> strain		
W303		<i>MATa/MATα his3-11,15/his3-11,15 can1-100/can1-100, leu2-3,112/leu2-3,112 trp1-1/trp1-1, ade2-1/ade2-1, ura3-1/ura3-1</i>

way have not been clearly identified, but many of the downstream signaling components are known (Berman and Sudbery, 2002). For example, production of cAMP by adenylyl cyclase plays a key role in inducing hyphal formation. In contrast to *Saccharomyces cerevisiae*, adenylyl cyclase is not needed for growth of *C. albicans* and instead plays an essential role in promoting hyphal formation (Rocha *et al.*, 2001). cAMP stimulation of protein kinase A results in phosphorylation of downstream targets, including the transcription factor Efg1, which is needed for hyphal growth (Stoldt *et al.*, 1997; Tebarth *et al.*, 2003). In addition, MAP kinase signaling activates the Cph1 transcription factor that is also involved in hyphal signaling (Lo *et al.*, 1997). Hyphal growth can also be promoted by proteolytic activation of the Rim101 transcription factor at alkaline pH (Davis *et al.*, 2000). The hyphal pathway is also negatively regulated by the repressor Tup1 (Braun and Johnson, 1997).

To better define the upstream components involved in hyphal signaling, as well as the differences between buds and hyphae, we carried out a proteomic comparison of plasma membrane proteins from budding and hyphal cells. GlcNAc was selected as the inducer of hyphal growth to better understand its role in this process, because the teleological basis for the effects of GlcNAc has not been understood from previous studies. GlcNAc was first reported to induce hyphae over 30 years ago in a screen for chemically defined inducers of hyphal growth in vitro (Simonetti *et al.*, 1974). Subsequent studies revealed *C. albicans* is capable of taking up GlcNAc and using it as an energy source, whereas the nonpathogenic *S. cerevisiae* lacks the proteins needed to transport and catabolize GlcNAc (Kumar *et al.*, 2000; Singh *et al.*, 2001). Mutational analysis indicates that the enzymes needed to catabolize GlcNAc in *C. albicans* also contribute to virulence (Singh *et al.*, 2001; Yamada-Okabe *et al.*, 2001). These include the enzymes that phosphorylate (Hxk1), deacetylate (Dac1), and deamidate (Nag1) GlcNAc, leading to its conversion to fructose-6-PO₄ (Kumar *et al.*, 2000; Singh *et al.*, 2001; Yamada-Okabe *et al.*, 2001). The GlcNAc transporter had not been identified previously, but in this study we report that one of the proteins identified by mass spectrometry (Ngt1) acts as a GlcNAc transporter, thus permitting direct analysis of its role in GlcNAc transport and signaling. Ngt1 is also significant in that it represents the first eukaryotic GlcNAc transporter to be identified and will therefore facilitate the analysis of the role of the homologous proteins in other organisms for their role in cellular regulation by GlcNAc.

MATERIALS AND METHODS

Strains and Media

The *C. albicans* and *S. cerevisiae* strains used in this study are described in Table 1. Strains were propagated on rich YPD medium or on synthetic medium essentially as described (Sherman, 1991). Uridine, 80 mg/l, was added to *C. albicans* cultures. To induce hyphal growth with different concentrations of GlcNAc, cells grown overnight in basal yeast medium (BYNB) containing galactose and buffered to pH 6.8 with 10 mM PIPES were washed and inoculated into 5 ml fresh medium at a density of 2×10^6 cells/ml. After 5 h of growth, the cells were washed with buffered BYNB medium and inoculated into buffered BYNB containing different concentrations of GlcNAc. Cells were grown at 37°C for 2 h, and then the cell morphologies were examined microscopically. The buffered medium and the growth regimen described above were used to maintain a low spontaneous level of hyphal formation that can be seen under other conditions due to farnesol depletion and pH changes when adjusting the cultures (Enjalbert and Whiteway, 2005).

The *C. albicans* NGT1-GFP strain was constructed by homologous recombination. PCR was used to add ~70 base pairs of sequence homologous to the 3' end of the NGT1 open reading frame (ORF) to a cassette that contains GFP and a URA3 selectable marker (Gerami-Nejad *et al.*, 2001). Ura⁺ colonies resulting from the transformation of the PCR product into *C. albicans* were then grown on GlcNAc medium and screened for GFP-positive cells by fluorescence microscopy. The *ngt1Δ* homozygous deletion strain was constructed by successive transformation of strain BWP17 with PCR-generated constructs containing either the ARG4 or HIS1 selectable marker genes flanked by ~70-base pair regions that were homologous to the sequences flanking the ORF of NGT1 (Wilson *et al.*, 1999). Proper integration of the GFP and deletion cassettes was verified by PCR.

Mass Spectrometry Analysis of Plasma Membrane Proteins

Cells were grown in budding phase by diluting a fresh overnight culture to 8×10^5 cells/ml in YPD medium and then incubating at 30°C until the cultures reached 10^7 cells/ml. To harvest cells initiating hyphal phase growth (germ tubes), a fresh overnight culture grown in YPD was diluted to 8×10^5 cells/ml in YPD medium and then grown to 10^7 cells/ml at 30°C. Cells were washed, resuspended in minimal medium containing 2.5 mM GlcNAc, and incubated at 37°C for 75–90 min. Germ tube emergence was confirmed by microscopy. Budding cells and germ tubes were harvested by centrifugation and washed with water, and then pellets of 10^9 cells were frozen for later use. The cell pellets were lysed by agitation with glass beads in cold TNE buffer with protease inhibitors (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 2.5 mM benzamide, and 30 μM pepstatin). The plasma membrane fraction was then isolated by density gradient centrifugation, essentially as described previously (Schandel and Jenness, 1994; Dosil *et al.*, 1998). In brief, cell extract was loaded at the bottom of a density gradient that ranged from 38 to 22% Renocal-76 (Bracco Diagnostic, Princeton, NJ). Tubes were spun in a Beckman SW40Ti rotor (Fullerton, CA) for 37 h at 35,000 rpm. The gradient was partitioned into 14 fractions by taking samples from the top, and then a 10-μl aliquot of each sample was run on a Western blot and probed with anti-Pma1 antibodies in order to detect the plasma membrane fraction. The plasma membrane fractions were subsequently extracted with ice-cold 1% Triton X-100 in order to isolate the detergent-resistant membrane fraction and thereby purify plasma membrane proteins away from contaminating cytoplasmic proteins, such as the abundant ribosomal proteins. Plasma membrane fractions extracted with cold 1% Triton X-100 were loaded at the bottom of a 40–5% sucrose gradient and spun in a SW41Ti rotor for 25 h at 35,000 rpm.

The gradient was split into different fractions and run on Western blots probed with anti-Pma1 antibodies to identify the detergent resistant membrane fraction that floated to the top of the gradient, which was then removed for analysis.

Approximately 500 µg of detergent-resistant membrane fraction from budding or germ tube cells was then separated by SDS-PAGE and prepared for mass spectrometry. 10-kDa ranges of the gel lanes were cut out, diced up, washed with 50% (vol/vol) methanol and 5% (vol/vol) acetic acid, dehydrated with acetonitrile, rehydrated with 10 mM DTT to reduce the samples, and then treated with 100 mM iodoacetamide to alkylate the proteins. The gel slices were washed with 100 mM ammonium bicarbonate, dehydrated with acetonitrile, and then dried. The samples were then rehydrated in 100 mM ammonium bicarbonate buffer and then 30 µl of 20 ng/ml sequencing grade TPCK-treated trypsin (Promega, Madison, WI) was added, and samples were incubated overnight at 37°C. Tryptic peptides were recovered with extraction buffer (50% acetonitrile and 50% formic acid) and analyzed at the Stony Brook University Proteomics Center by liquid chromatography followed by tandem mass spectrometry with electrospray ionization (nano-LC/ESI/MSMS) on an API Qstar Pulsar i quadrupole-time of fly (Q-TOF) tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Mass spectrometry results were then compared with the ORF19 release of the *Candida* genome database to identify the corresponding proteins (<http://www.candidagenome.org/>).

Plasmid Construction

A plasmid carrying *NGT1* was constructed by PCR amplification of the genomic sequence from 974 base pairs upstream of the initiator ATG to 354 bases downstream of the terminator codon. This DNA fragment was then inserted between the *SacI* and *SacII* restriction sites of the *URA3* plasmid pDDB57 (Wilson *et al.*, 2000). The resulting plasmid was linearized in the promoter region by digestion with *EcoRV*, and integrated into the *ngt1Δ* strain YJA2 to create a complemented strain called YJA4. A plasmid designed to express *NGT1* in *S. cerevisiae* under control of the *GAL1* promoter was constructed by PCR amplifying the *NGT1* ORF and inserting it at the *SpeI* site in the polylinker region of plasmid pRS426GAL1 (Mumberg *et al.*, 1994).

GlcNAc Uptake Assays

To assay GlcNAc uptake in *C. albicans* strains, overnight cultures were harvested by centrifugation, washed in BYNB, and then inoculated into fresh BYNB containing either dextrose or GlcNAc. Cells were grown for 4 h at 30°C to induce the GlcNAc pathway, harvested by centrifugation, washed with BYNB, and then resuspended at 1.1×10^8 cells/ml. Uptake assays were carried out in 96-well plates and were initiated by adding 10^7 cells to 10 µl of radioactive [3 H]GlcNAc/well (200 µM final concentration; 5 mCi/mmol). Reactions were terminated by collecting cells onto Whatman GF/C glass microfiber filters (Clifton, NJ) using a Brandel Cell Harvester (Gaithersburg, MD). Filters were dried, and then the [3 H]GlcNAc taken up by the cells was quantified using a scintillation counter.

To assay GlcNAc uptake in *S. cerevisiae* strains, W303 cells containing either pRS426GAL1-*NGT1* or the control empty vector were grown overnight in synthetic medium lacking uracil and containing either 2% dextrose or galactose. Cells were then washed, inoculated into fresh medium, and grown for 4 h at 30°C. [3 H]GlcNAc uptake assays were then carried out as described above. The ability of different sugars to compete for the uptake of GlcNAc was assayed in *S. cerevisiae* strain W303 containing pRS426GAL1-*NGT1* that was prepared as described above. Cells, 10^7 , were added per well of 96-well plates that also contained [3 H]GlcNAc (200 µM final) plus either 2 mM (10×) or 20 mM (100×) final concentration of one of the following cold competitor sugars: GlcNAc, dextrose, galactose, fructose, glucosamine or *N*-acetylmannosamine.

Macrophage Infection Assay

J774 macrophage cells were prepared for infection by plating 10^5 cells/well on coverslips in 24-well plates containing DMEM medium with 10% FBS. Cells were incubated overnight and then the medium was replaced with fresh DMEM lacking FBS. The *C. albicans* strains were prepared for infection assays by growing overnight in YPD, and then washing and resuspending the cells in phosphate-buffered saline (PBS) at 10^7 cells/ml. Infections were initiated by adding the appropriate *C. albicans* strain to a well of J774 cells at an MOI of 1. Cells were incubated for 40 min at 37°C, and then the J774 cells were washed and fresh medium was added. Assays were stopped at different time points by adding paraformaldehyde to 2.5% final concentration for 20 min. Samples were then washed, mounted on slides, and examined microscopically as described below.

Microscopy

Ngt1-GFP fluorescence was analyzed in cells that were grown overnight in synthetic complete medium containing glucose but lacking uracil. Cells were washed with BYNB and then incubated with minimal medium containing different sugars or serum for 2–4 h at 37°C. Cells were washed with BYNB and analyzed immediately by microscopy. To compare the morphogenesis of wild-type versus *ngt1Δ* cells, cells were grown overnight in synthetic medium containing dextrose, washed, adjusted to $2-3 \times 10^5$ cells/ml in BYNB, and

incubated in the presence or absence of 2 mM GlcNAc at 37°C. Cells were analyzed by fluorescence microscopy to detect GFP and by differential interference (DIC) microscopy to detect cell morphology. Images were captured using an Olympus BH2 microscope (Melville, NY) equipped with a Zeiss AxioCam digital camera (Thornwood, NY). Fluorescence intensity of the images was quantified using Openlab 3.0.8 software from Improvision (Lexington, MA).

Analysis of NGT1 Homologues

BLAST searches (Altschul *et al.*, 1990) were carried out to identify *NGT1* homologues in the genome sequences of other organisms present at the Web site of the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignments of the predicted Ngt1 proteins and an evolutionary tree of their relatedness were carried out using Clustal W (Thompson *et al.*, 1994).

RESULTS

Identification of GlcNAc-induced Plasma Membrane Proteins by Mass Spectrometry

A proteomics approach aimed at identifying plasma membrane proteins that are unique to the bud or hyphal phases of *C. albicans* was carried out to identify new proteins related to the regulation of morphogenesis and virulence. Plasma membranes were purified by density gradient centrifugation from budding cells grown in standard medium (YPD) or from cells induced with GlcNAc to form hyphae. The detergent-resistant fraction of the plasma membrane was then isolated to help remove contaminating cytoplasmic proteins (see *Materials and Methods*). The purified membrane proteins were then separated by SDS gel electrophoresis, trypsin-digested, and analyzed by liquid chromatography followed by tandem mass spectrometry. Comparison of the mass spectrometry data with the ORFs predicted in the *C. albicans* genome sequence identified 137 different proteins. Approximately one third were detected only in buds, one third in hyphal cells, and one third were common to both buds and hyphae. A representative set of the proteins that were identified is shown in Table 2, and the full set of proteins is described in the Supplementary Data.

Transporters comprised the major class of proteins, which accounted for 40% of the total. The majority of the proteins were thus membrane-spanning proteins, although ~5% of the proteins were GPI anchored and another 5% were acylated. Another major group of proteins were those involved in iron uptake (~5%), which is critical for *C. albicans* virulence. About 15% of the proteins did not have an obvious homolog in *S. cerevisiae*, and a similar fraction did not have an obvious prediction for their function. Most types of proteins were represented at roughly similar levels in the different groups. However, it was interesting that the proteins lacking a homolog in *S. cerevisiae* represented 19% of those found only in buds, 18% of those found in germ tubes, but only 6% of those found in both buds and germ tubes. Altogether there were 20 proteins that lack a homolog in the nonpathogenic *S. cerevisiae* and are thus candidates for carrying out virulence functions in *C. albicans*.

Ngt1-GFP Is Specifically Induced by GlcNAc

A protein we named Ngt1 that corresponds to ORF orf19.5392 in the *C. albicans* genome was selected for further study, because it was identified with very high confidence by mass spectrometry and initially appeared to be specific to hyphae. Subsequent analyses detected Ngt1 in budding cells, albeit with lower confidence scores, suggesting that Ngt1 was likely to be induced by GlcNAc. Ngt1 is also predicted to contain multiple transmembrane domains and was therefore unlikely to be a cytoplasmic contaminant. It was also interesting that homologues of Ngt1 were absent from *S. cerevisiae* and its close relatives, but present in a wide

Table 2. Representative subset of proteins identified by mass spectrometry

Cell type	ORF19 ^a	<i>C. albicans</i> gene	<i>S. cerevisiae</i> homolog ^b	Description of function ^c
Bud	5759	SNQ2	SNQ2	ABC transporter protein involved in multidrug resistance
	1415	FRE10	FRE1	Cell-surface ferric reductase
	4118	CNT	None	Cation-coupled nucleoside transporter
	3765	RAX2	RAX2	Transmembrane protein involved in bipolar bud site selection
Germ tube	7001	—	YCK1,2	Casein kinase I
	7094	HGT12	SNF3	MFS superfamily glucose sensor/transporter
	4335	TNA1	TNA1	Nicotinic acid transporter
	2614	RSR1	RSR1	RAS-related protein
	176	OPT4	OPT2	Oligopeptide transporter
	7030	SSR1	CCW14	GPI anchor protein with a role in cell wall structure
Both	5383	PMA1	PMA1,2	Plasma membrane H(+)-ATPase
	4456	GAP4	GAP1	Putative amino acid permease; hyphal induced
	3829	PHR1	GAS1,2	GPI-anchored glycoside; role in cell wall structure
	3646	CTR1	CTR1	Copper transporter
	1760	RAS1	RAS1,2	RAS signal transduction GTPase

^a Open reading frame designation from ORF19 release of the *C. albicans* genome database.

^b Closest homolog found in the *S. cerevisiae* genome.

^c Function as indicated by experimental work or by close similarity to known genes in other organisms.

range of other fungi, including the pathogens *Cryptococcus neoformans* and *Aspergillus fumigatis*, suggesting that Ngt1 could be related to virulence.

To verify that Ngt1 encodes a plasma membrane protein present in GlcNAc-induced hyphal cells, an *NGT1-GFP* fusion gene was constructed in the genome of *C. albicans* by introducing a GFP tag at the C terminus. Fluorescence microscopy readily detected Ngt1-GFP at the periphery of GlcNAc-induced hyphae, consistent with plasma membrane localization. In contrast, Ngt1-GFP was not detected in budding cells grown in glucose medium (Figure 1). Ngt1-GFP was also not detected in cells that were induced to form hyphae by treatment with serum, one of the strongest known inducers of hyphal morphogenesis. These results indicate that *NGT1* is induced by GlcNAc and is not generally a hyphal-induced gene.

The specificity of *NGT1* regulation was analyzed further by growing cells in medium containing different sugars

(Figure 2A). Ngt1-GFP was only highly induced by GlcNAc and not by other related sugars such as glucose, galactose, fructose, glucosamine, or *N*-acetylmannosamine. Quantitation of the fluorescence signal indicated that Ngt1-GFP was induced >50-fold in GlcNAc medium. Growth of the cells in medium containing a second sugar in addition to GlcNAc demonstrated that glucose strongly repressed the expression of *NGT1*. The glucose repression was not complete as very weak Ngt1-GFP fluorescence could be detected above the background seen in cells grown only in glucose (Figure 2B). Galactose, fructose, glucosamine and *N*-acetylmannosamine still permitted high-level induction of Ngt1-GFP, although they did cause a partial reduction in Ngt1-GFP fluorescence. Thus, *NGT1* is specifically induced by GlcNAc and is repressed by glucose.

Ngt1-GFP Is Induced by Macrophage Phagocytosis

The role of GlcNAc signaling in *C. albicans* has been unclear, because GlcNAc was initially identified as an inducer of hyphal growth in a screen for a chemically defined alternative to serum (Simonetti *et al.*, 1974). Therefore, we took advantage of *NGT1-GFP* as a reporter gene to examine a possible biological role for GlcNAc signaling. In particular, the ability of macrophage phagocytosis to induce Ngt1-GFP was examined because phagocytosis is known to induce hyphal growth (Lo *et al.*, 1997; Lorenz *et al.*, 2004). Phagolysosomes are also low in glucose and are likely to contain GlcNAc due to the degradation carbohydrate chains on glycoproteins or as a result of the cleavage of *Candida* cell wall chitin by acidic mammalian chitinase to release the GlcNAc subunits (see *Discussion*). As shown in Figure 3, Ngt1-GFP was induced after phagocytosis by the murine macrophage cell line J774. Although there was detectable nonspecific fluorescence in J774 cells that had phagocytosed untagged wild-type cells, the level of Ngt1-GFP was readily detected above this background in three independent experiments. The Ngt1-GFP fluorescence was also qualitatively different in that it appeared more evenly across the plasma membrane than did the nonspecific fluorescence. Consistent with this, microarray analysis of gene expression found that

Figure 1. Ngt1-GFP induction by GlcNAc. *C. albicans* strain YJA1 carrying an *NGT1-GFP* fusion gene was grown in the indicated medium for 2 h at 37°C, and then fluorescence and light microscope images were recorded. Cells were incubated in minimal medium containing glucose, GlcNAc, or glucose plus 10% bovine serum as indicated. Fluorescent microscope images of Ngt1-GFP are shown in the top panels and light microscope (DIC) images are shown below. Bar, 10 μ m.

Figure 2. NgT1-GFP is specifically induced by GlcNAc and is repressed by glucose. (A) Relative fluorescence of cells incubated in medium containing the indicated sugar for 4 h. For each sugar, the first bar represents the *NGT1-GFP* strain YJA1, and the second bar is the untagged strain SC5314. Fluorescence was very low under all conditions for the untagged strain. (B) Relative fluorescence of cells induced with the indicated sugar or combination of GlcNAc and another sugar. Note that glucose repressed induction of NGT1-GFP. Relative fluorescence was determined from analysis of digital microscope images (see *Materials and Methods*).

NGT1 and the genes needed for GlcNAc catabolism are induced after phagocytosis (Lorenz *et al.*, 2004). These results indicate that the GlcNAc pathway is activated after phagocytosis and suggest that one aspect of the biological role for the response to GlcNAc is to signal cells to form hyphae after phagocytosis.

NGT1 Is Required for Efficient Growth on GlcNAc and Hyphal Induction

Hydropathy analysis of the NgT1 protein indicates that it contains 12 transmembrane domains, which is characteristic of transporter proteins and suggests that NgT1 may act as a GlcNAc transporter. To test this possibility, a homozygous deletion strain was constructed in which both copies of *NGT1* were deleted (*C. albicans* is a diploid organism). The deletion mutations were constructed in the BWP17 strain (Wilson *et al.*, 1999) by replacing one allele of *NGT1* with *ARG4* and the other allele with *HIS1*. As shown in Figure 4, the *ngt1Δ* mutant grew normally in glucose medium, but was defective for growth in the GlcNAc medium commonly used for hyphal induction (2.5 mM GlcNAc).

The *ngt1Δ* mutant was also defective in forming hyphae under the standard conditions of 2.5 mM GlcNAc. The defect was examined further by testing whether cells can form hyphae at higher concentrations of GlcNAc. The rationale for this is based on the previous observation that cells with

Figure 3. NgT1-GFP is induced after macrophage phagocytosis. The murine macrophage cell line J774 was infected with *C. albicans* NGT1-GFP strain YJA1 or wild-type strain SC5314 at an MOI of 1 for 40 min. The cells were incubated for 2 h and then fixed with paraformaldehyde. Fluorescent microscope images of NgT1-GFP are shown on top and light microscope (DIC) images are shown below. Bar, 10 μ m.

a mutation in the galactose transporter can grow at high concentrations of galactose, but not at lower doses that are still permissive for growth of wild-type cells (Douglas and Condie, 1954). Alternatively, some transporter-type proteins have been found to transduce a separate signal pathway (e.g., glucose or ammonium; Holsbeeks *et al.*, 2004; Wu *et al.*, 2006). To distinguish between these possibilities, cells were examined after growth in different concentrations of GlcNAc. Interestingly, *ngt1Δ* mutants started forming hyphae in response to 100 mM GlcNAc, which was ≥ 1000 -fold higher than the concentration required for wild-type cells to form hyphae (Figure 5). GlcNAc, 100 mM, also caused clumping of the *ngt1Δ* cells, which indicates that the adhesin proteins were induced in the hyphal cell walls (Sundstrom, 2002). Hyphal formation at 100 mM was still specific to GlcNAc and was not seen with dextrose or galactose (data not shown). GlcNAc, 100 mM, is not an unusually high level of sugar; yeast medium typically contains ~ 111 mM dextrose (2% wt/vol). The mutant phenotypes were complemented by reintroduction of a wild-type copy of *NGT1*, and the

Figure 4. *NGT1* is required for growth on GlcNAc medium. Wild-type strain SC5314, *ngt1Δ* homozygous deletion strain YJA3, and strain YJA4 in which *ngt1Δ* was complemented by reintroduction of *NGT1*, were adjusted to 5×10^6 cells/ml, and then 10-fold serial dilutions of cells were spotted onto solid medium plates containing either glucose or GlcNAc. The plates were incubated for 2 d at 30°C and then photographed.

Figure 5. High levels of GlcNAc can bypass the role of Ngt1 in stimulating hyphal growth. Wild-type strain DIC185, *ngt1Δ* homozygous deletion strain YJA3, and strain YJA4 in which *ngt1Δ* was complemented by reintegration of *NGT1* were grown to log phase, resuspended in medium containing the indicated concentration of GlcNAc, and then incubated at 37°C for 2 h. Hyphal cells clumped, presumably due to induction of the cell surface adhesin proteins. Bar, 10 μ m.

ngt1Δ mutant could form hyphae efficiently in response to serum, which demonstrate that the effects of this mutation are specific to the GlcNAc response.

NGT1 Is Required for GlcNAc Transport in C. albicans

To gain further support for the idea that Ngt1 mediates GlcNAc transport, we examined the ability of the mutant cells to take up radioactive GlcNAc. Previous studies have demonstrated that glucose-grown cells do not display significant GlcNAc uptake and that cells must first be pregrown in GlcNAc for several hours to induce the transporter function (Singh and Datta, 1978, 1979). Therefore, we analyzed the ability of wild-type and of *ngt1Δ* cells to take up [3 H]GlcNAc after being incubated for 4 h in medium containing either glucose or GlcNAc. Cells were then washed and resuspended in basal yeast medium without sugar (BYNB). Aliquots of cells were incubated for different times with [3 H]GlcNAc and then rapidly harvested by filtration. Quantitation of the results by scintillation counting clearly demonstrated that GlcNAc-grown wild-type cells take up GlcNAc very efficiently, but that the *ngt1Δ* mutants only took up very low levels of GlcNAc (Figure 6). Complementation of the *ngt1Δ* mutation by reintroducing a wild-type copy of *NGT1* restored the ability to take up GlcNAc with high efficiency.

NGT1 Promotes GlcNAc Uptake When Expressed in S. cerevisiae

To more directly confirm that Ngt1 mediates GlcNAc uptake, we took advantage of the fact that *S. cerevisiae* lacks the ability to transport GlcNAc (Singh and Datta, 1979). The *NGT1* gene was therefore placed under control of the galactose-regulated *GAL1* promoter in plasmid p426GAL1 and transformed into *S. cerevisiae* strain W303. Cells were then cultured in glucose or galactose medium and then prepared for GlcNAc uptake assays. The results demonstrated that *S. cerevisiae* cells carrying the *GAL1-NGT1* plasmid rapidly took up high levels of GlcNAc when grown in galactose medium (Figure 7A). Significant uptake was even observed in samples that were processed quickly for the time zero control. In contrast, cells that were grown in glucose to repress *NGT1* did not display significant uptake of GlcNAc. In addition, cells carrying the empty vector control did not show significant GlcNAc uptake when pregrown in either medium (Figure 7B). As an additional control, an *NGT1-GFP* fusion gene was expressed in *S. cerevisiae*, and the GFP fluorescence was detected primarily at the plasma mem-

Figure 6. *NGT1* is needed for efficient GlcNAc uptake by *C. albicans*. (A) Wild-type strain SC5314, (B) *ngt1Δ* homozygous deletion strain YJA3, and (C) strain YJA4 in which *ngt1Δ* complemented by reintegration of *NGT1* were incubated for 4 h at 30°C in minimal medium containing GlcNAc ($\blacktriangle$) to induce *NGT1*, and were also grown in glucose medium ($\blacksquare$) as a control. Cells were harvested and washed, and then 10^7 cells were then incubated in 200 μ M [3 H]GlcNAc for the indicated time. Cells were collected on filters, and the [3 H]GlcNAc taken up was determined by scintillation counting. Error bars, SE.

Figure 7. NGT1 expression in *S. cerevisiae* is sufficient to promote GlcNAc uptake. *S. cerevisiae* strain W303 carrying a plasmid designed to (A) express NGT1 under control of the galactose-inducible promoter *GAL1* or (B) the empty vector plasmid (pRS426GAL1). Cells were grown in galactose medium (▲) overnight to induce NGT1, or in glucose medium (■) to repress NGT1. Cells ($n = 10^7$) were incubated with $200 \mu\text{M}$ [^3H]GlcNAc for the indicated time and then the [^3H]GlcNAc taken up was determined by scintillation counting. (C) Fluorescent microscope image and corresponding light microscope image of *S. cerevisiae* cells induced with galactose to express NGT1-GFP. Variable levels of Ng1-GFP observed in different cells are expected due to the cells containing different levels of the multicopy plasmid vector pRS426GAL1 used to express NGT1-GFP. Error bars, SE.

brane (Figure 7C). Thus, expression of NGT1 in *S. cerevisiae* is sufficient to promote GlcNAc uptake, indicating that the Ng1 protein directly promotes the uptake of GlcNAc.

Ng1 Is Specific for GlcNAc Transport

The specificity of transport by Ng1 was examined by assaying the ability of various sugars to compete for the uptake of radioactive GlcNAc in *S. cerevisiae* cells that were induced to express NGT1. For this analysis, cells were incubated in a reaction mix that contained $200 \mu\text{M}$ [^3H]GlcNAc and either a 10-fold (2 mM) or 100-fold (20 mM) excess of a nonradioactive competitor sugar. Addition of a 10-fold excess of nonradioactive GlcNAc was sufficient to strongly reduce the uptake of the [^3H]GlcNAc, as expected (Figure 8). However, addition of even 100-fold excess of other competitor sugars did not have significant effects on [^3H]GlcNAc uptake. The competitor sugars tested were closely related to GlcNAc and

Figure 8. Ng1 is specific for GlcNAc uptake. The specificity of the transport activity promoted by Ng1 was examined by testing the ability of other sugars to compete with the uptake of [^3H]GlcNAc. Assays were carried out with *S. cerevisiae* strain W303 that expressed NGT1 under control of the galactose-inducible promoter *GAL1* as described in the legend to Figure 7. Reactions contained $200 \mu\text{M}$ [^3H]GlcNAc plus either 2 mM or 20 mM of the indicated nonradioactive sugar and were carried out for 2 min. The mock sample received only water. The results demonstrate that only nonradioactive GlcNAc, and not other related sugars, competed for the uptake of [^3H]GlcNAc. Error bars, SE.

included glucose, galactose, fructose, glucosamine, and N-acetylmannosamine. These results indicate that Ng1 is a specific transporter for GlcNAc.

Ng1 Homologues

Because there have been no previous reports of a eukaryotic GlcNAc transporter, we performed BLAST searches and found Ng1 homologues in diverse organisms including plants and metazoans. Interestingly, *C. albicans* Ng1 showed much higher conservation with the homologues from the plants *Arabidopsis thaliana* and *Oryza sativa* (>41% identity) than it did with the metazoan homologues ($\leq 25\%$ identity). The metazoan homologues were mainly conserved between residues 100–235, which includes transmembrane domains 2–6 that play a major role in solute recognition in the lacY lactose transporter (Abramson *et al.*, 2004). The closer similarity of fungal and plant Ng1 homologues was very unexpected, because fungi are much more closely related to metazoans than they are to plants (Figure 9; James *et al.*, 2006). This suggests that there may have been selective pressure to maintain the similarity of Ng1 in plants and fungi. Alternatively, it is intriguing to speculate that there may have been horizontal gene transfer. The plant Ng1 homologues clustered most closely with those from plant pathogenic fungi (e.g., *Ustilago maydis*) in a phylogenetic tree generated by Clustal W.

Analysis of the Fungal Kingdom indicates that the ability to utilize GlcNAc was lost independently in several lineages. Ng1 homologues were detected in three of the five major divisions of the fungi (Figure 9). In the Ascomycota division, NGT1 appears to have been lost in two out of three of the classes. In the Saccharomycotina class that includes the budding yeasts (e.g., *C. albicans* and *S. cerevisiae*), the 6 organisms most closely related *C. albicans* contain an NGT1 homologue, and all 10 yeast that were more related to *S. cerevisiae* lack an NGT1 homologue. A similar pattern was detected for the GlcNAc catabolic enzymes DAC1 and NAG1. The loss of NGT1 coincides with an evolutionary split that occurred before the appearance of the triplicated mating cassettes and short centromeres but after the appearance of retrotransposons Ty3

Figure 9. Evolutionary conservation of *NGT1*. BLAST searches (Altschul *et al.*, 1990) were used to identify *NGT1* homologues in the genome sequences of other organisms. White letters in black boxes indicate the presence of *NGT1* and black letters indicate the absence. **S. kluyveri* is marked by an asterisk because it lacks *NGT1* and *DAC1*, but contains *NAG1*. ***S. pombe* is marked because it contains an *NGT1* homolog but lacks the other catabolic genes (e.g., *DAC1* and *NAG1*). ****P. carinii* contains an *NGT1* homolog, but the genome sequence is

not complete so it is not clear if homologues of *NAG1* and *DAC1* will be present. The evolutionary tree was constructed based on a recent phylogenetic analysis of fungi (James *et al.*, 2006) and also on an analysis of Ascomycota (Dujon, 2006). The branches in the figure were sized to clearly display the relationship of the different lineages and are not sized to represent predicted evolutionary distance.

and Ty5 (Dujon, 2006). In a second class of the Ascomycota (Taphrinomycotina), an *NGT1* homolog was detected in the fission yeast *Schizosaccharomyces pombe* but not in the related *Schizosaccharomyces japonica*. Neither contains homologues of the GlcNAc catabolic genes, and *S. pombe* was not able to grow in GlcNAc medium. In contrast, an *NGT1* homolog was readily detected in all 22 members of the Pezizomycotina class (e.g., *A. fumigatis* and *Neurospora crassa*), which includes many plant and animal pathogens. Although many pathogenic fungi contain *NGT1*, the correlation is not 100% because the human pathogen *Candida glabrata* and the plant pathogen *Ashbya gossypii* lack homologues for *NGT1* and the GlcNAc catabolic genes.

DISCUSSION

GlcNAc plays important roles in cell signaling in a wide range of cells in addition to serving as a source of nutrition. In metazoans, GlcNAc is a sensor of nutrient status that is involved in insulin signaling, cell cycle control, and other essential processes. Modulation of GlcNAc levels promotes O-linked attachment of GlcNAc to serine or threonine residues on proteins as a regulatory modification that is analogous to phosphorylation (Slawson and Hart, 2003; Zachara and Hart, 2006). In *C. albicans*, GlcNAc induces the formation of hyphal cells, which is an underlying virulence trait as budding and hyphal cells are thought to carry out unique roles during infection. In addition to different morphologies, budding and hyphal cells differ in the expression of virulence factors (Nantel *et al.*, 2002; Lorenz *et al.*, 2004), such as adhesin proteins, secreted hydrolytic enzymes, and antioxidant enzymes (Hube, 2004; Whiteway and Oberholzer, 2004; Kumamoto and Vines, 2005). Therefore, in this study we used mass spectrometry to identify plasma membrane proteins in budding cells and GlcNAc-stimulated hyphae, which have the potential to mediate interaction with the host environment and contribute to the infectious process. One protein was selected for detailed analysis and was subsequently named *Ngt1* for its role as an *N*-acetylglucosamine transporter. *Ngt1* was also found to mediate GlcNAc-induced hyphal formation in *C. albicans*. These results are also significant in that *Ngt1* represents the first eukaryotic GlcNAc transporter to be identified. This will make it possible to examine the role of homologues present in a wide range of organisms from yeast to plants and animals for their function in GlcNAc transport and signaling.

Ngt1-GFP Induction by GlcNAc

Ngt1-GFP was specifically induced by GlcNAc, and not by the switch to hyphal growth, because *Ngt1*-GFP was not induced in serum-stimulated hyphae (Figure 1). In addition,

Ngt1-GFP was highly induced by GlcNAc at 23 or 30°C, even though *C. albicans* cells do not switch to hyphal growth under these lower temperature growth conditions (F.J.A. and J.B.K., unpublished data). Only GlcNAc induced *Ngt1*-GFP, and not other related sugars such as glucosamine and *N*-acetylmannosamine. Glucose repressed the ability of GlcNAc to induce *Ngt1*-GFP. Altogether these studies indicate that *NGT1* expression is specifically regulated by GlcNAc, similar to the enzymes needed for GlcNAc catabolism in *C. albicans* (Kumar *et al.*, 2000; Singh *et al.*, 2001; Yamada-Okabe *et al.*, 2001). However, *NGT1* is on Chromosome 6 and is thus not linked to the other catabolic genes (*NAG1*, *DACX1*, and *HXX1*), which are present in a cluster on Chromosome 3 (Kumar *et al.*, 2000; Yamada-Okabe *et al.*, 2001). Attempts to identify a common DNA sequence motif in the upstream regions of these genes using MEME (Bailey and Elkan, 1994) were inconclusive.

Ngt1 Functions as a GlcNAc Transporter

The predicted amino acid sequence of *Ngt1* indicates that it is a transporter of the major facilitator superfamily (MFS; Pao *et al.*, 1998; Abramson *et al.*, 2004). One hallmark of MFS transporters is that they contain 12 transmembrane domains. Hydrophathy analysis of *Ngt1* readily identified 11 transmembrane domains, and a twelfth transmembrane domain was identified as an amphipathic helix in which the polar side presumably faces the helix bundle to shield it from the nonpolar membrane environment. *Ngt1* also contains a close match to the consensus sequence (GTXXNXXGXRXXL) that is commonly found in intracellular loop 1 of MFS transporters (Pao *et al.*, 1998).

A homozygous *ngt1Δ* deletion mutant was defective in growing on GlcNAc medium (Figure 4) and in GlcNAc transport (Figure 6). Although this implicated *Ngt1* as a GlcNAc transporter, it was not clear from these studies whether *Ngt1* is directly involved in transport. In *S. cerevisiae*, the *Snf3* and *Rgt2* glucose sensors display sequence similarity to transporters, yet they are inactive as transporters and function instead as receptors that activate a signal pathway leading to induction of active glucose transporters (Moriya and Johnston, 2004; Kim and Johnston, 2006). Therefore, we took advantage of the fact that *S. cerevisiae* lacks the ability to transport GlcNAc (Singh and Datta, 1978, 1979) to use it as a heterologous expression system. *S. cerevisiae* cells expressing *NGT1* displayed rapid GlcNAc uptake that was similar to the levels seen in *C. albicans*. The specificity of *Ngt1* transport function was confirmed in a competition assay in which closely related sugars such as glucosamine and *N*-acetylmannosamine did not outcompete the uptake of [³H]GlcNAc. These results, combined with the similarity

of Ngt1 to MFS transporters, demonstrate that Ngt1 is a GlcNAc-specific transporter.

Role of Ngt1 in Hyphal Growth Stimulated by GlcNAc

The initial observation that *ngt1Δ* cells were defective in forming hyphae under standard GlcNAc induction conditions (~2 mM) demonstrated that Ngt1 is involved in promoting this morphological change. However, it was not clear from this whether Ngt1 acts only as a transporter and that cells respond to increased intracellular levels of GlcNAc or if Ngt1 activates a separate signal pathway to induce hyphal formation. Many transporter proteins have been shown recently to also act as signal transducers (Holsbeeks *et al.*, 2004; Moriya and Johnston, 2004; Wu *et al.*, 2006). For example, the *C. albicans* Mep2 ammonium permease activates the Cph1 MAP kinase and the cAMP kinase pathways that induce hyphal growth (Biswas and Morschhauser, 2005). However, it is not necessary for Ngt1 to transduce a separate signal. An *ngt1Δ* mutant could be induced to form hyphae by exposing cells to 1000-fold higher concentrations of GlcNAc than were required to stimulate hyphal formation in the wild type (Figure 5), indicating that cells are responding to increased intracellular levels of GlcNAc. GlcNAc uptake under these conditions presumably occurs via a low-affinity process that has been observed in *C. albicans* (Singh and Datta, 1978). Thus, it appears that the primary role of Ngt1 is to facilitate high-affinity transport of GlcNAc.

The ability of *C. albicans* cells to form hyphae in response to elevated GlcNAc, but not to exogenously added glucosamine or fructose, suggests that the intracellular signal is an upstream component such as GlcNAc itself. GlcNAc taken up by *C. albicans* is converted to GlcNAc-6-PO₄ and then deacetylated to glucosamine-6-PO₄ and deamidated to fructose-6-PO₄. One model for how GlcNAc signaling may occur is based on galactose regulation in *S. cerevisiae*. Cells respond to elevated intracellular galactose, which acts together with ATP and Gal3 to relieve the inhibition of the Gal4 transcription factor by its negative regulator Gal80 (Sil *et al.*, 1999). Another possibility is based on GlcNAc signaling in metazoan cells, in which elevated GlcNAc levels stimulate signal transduction via O-GlcNAc modification of proteins (Slawson and Hart, 2003; Zachara and Hart, 2006). However, this modification has not been reported in fungi, so it remains to be determined how the GlcNAc signal is propagated in *C. albicans*.

Role of Ngt1 during Macrophage Phagocytosis

The biological role for GlcNAc signaling in *C. albicans* has not been well defined; the ability of GlcNAc to induce hyphae was discovered in a search for a chemically defined inducers in vitro (Simonetti *et al.*, 1974). Subsequent studies revealed that the GlcNAc catabolism genes (*HXX1*, *DAC1*, and *NAG1*) contribute to the virulence of *C. albicans* in a mouse tail-vein injection model of pathogenesis (Singh *et al.*, 2001; Yamada-Okabe *et al.*, 2001). The GlcNAc catabolic genes are repressed in serum, which contains glucose, but *C. albicans* also grows in low glucose environments such as the macrophage phagolysosome (Lorenz *et al.*, 2004). Interestingly, Ngt1-GFP was induced after macrophage phagocytosis (Figure 3). *NGT1* and the genes needed for GlcNAc catabolism were also found to be induced after macrophage phagocytosis by microarray analysis of gene expression (Lorenz *et al.*, 2004). The correlation between GlcNAc induction and phagocytosis is also strengthened by the observation that 6 of the 10 GlcNAc-induced proteins identified with highest confidence by mass spectrometry correspond to

genes that were induced by macrophage phagocytosis. However, the *ngt1Δ* mutant was still capable of forming hyphae after phagocytosis (F.J.A. and J.B.K., unpublished data), indicating either that GlcNAc levels are very high or that other hyphal inducers are present in the phagolysosome. Perhaps multiple signals stimulate hyphal growth after phagocytosis to ensure an effective response to the hostile phagolysosomal environment. The importance of evading host defenses may also explain why such a variety of different stimuli can induce hyphal growth in vitro. Future studies on GlcNAc signaling in *C. albicans* will help define how various stimuli are integrated to regulate hyphal growth and will also serve as a model for defining the role of GlcNAc regulation in other organisms.

Ngt1 Homologues

The identification of Ngt1 as a GlcNAc transporter made it possible to search for homologues in other organisms (Figure 9). Ngt1 homologues were present in at least 3 of the 5 divisions of the fungal kingdom. It appears that *NGT1* and the GlcNAc catabolic enzymes (*HXX1*, *DAC1*, and *NAG1*) were independently lost in several branches of the fungal lineage, including the branch between *S. cerevisiae* and *C. albicans* in the Ascomycota. The galactose utilization genes have also been lost several independent times in the Ascomycota, although the losses of the galactose and GlcNAc genes do not coincide (Dujon, 2006). Many human and plant fungal pathogens contain *NGT1* and the GlcNAc catabolic genes, but they are not required for all pathogens since *C. glabrata* and *A. gossypii* lack homologues for these genes.

NGT1 homologues were also detected in two very divergent branches of multicellular organisms, plants and metazoans. Surprisingly, *C. albicans* Ngt1 showed much higher conservation with the plants homologues, given that fungi are much more closely related to metazoans (James *et al.*, 2006). Current models assume that GlcNAc levels in metazoans are regulated indirectly by other nutritional effects. Thus, the discovery of Ngt1 homologues in metazoans broadens the possible functions of GlcNAc signaling to include intercellular communication. In this regard it is interesting that as part of a full-genome RNAi study in *Caenorhabditis elegans* interference of an Ngt1 homologue caused defects in early embryogenesis (Sonnichsen *et al.*, 2005). Other metazoan homologues have not been studied, but they are apparently expressed since they are represented in cDNA libraries. It will therefore be important to determine the role of Ngt1 homologues, given the essential role that GlcNAc signaling plays in biomedically important processes such as insulin signaling, cell cycle control, and proteosome regulation (Slawson and Hart, 2003; Zachara and Hart, 2004, 2006).

ACKNOWLEDGMENTS

We thank Scott Filler for providing plasmids, strains and advice; James Bliska, Hana Fukuto, and Kate Klein for assistance with growing J774 cells; and Lois Douglas for advice and comments on the manuscript. We also thank the reviewers for their helpful suggestions. This work was supported by Grant RO1 AI47837 from the National Institutes of Health that was awarded to J.B.K.

REFERENCES

- Abramson, J., Iwata, S., and Kaback, H. R. (2004). Lactose permease as a paradigm for membrane transport proteins. *Mol. Membr. Biol.* 21, 227–236.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.
- Bailey, T., and Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. In: *Proceedings of the Sec-*

- and International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA: AAAI Press, 28–36.
- Barnhart, M. M., Lynem, J., and Chapman, M. R. (2006). GlcNAc-6P levels modulate the expression of Curli fibers by *Escherichia coli*. *J. Bacteriol.* 188, 5212–5219.
- Berman, J., and Sudbery, P. E. (2002). *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat. Rev. Genet.* 3, 918–930.
- Biswas, K., and Morschhauser, J. (2005). The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*. *Mol. Microbiol.* 56, 649–669.
- Braun, B. R., and Johnson, A. D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. *Science* 277, 105–109.
- Chou, T. Y., and Hart, G. W. (2001). O-linked *N*-acetylglucosamine and cancer: messages from the glycosylation of c-Myc. *Adv. Exp. Med. Biol.* 491, 413–418.
- Davis, D. (2003). Adaptation to environmental pH in *Candida albicans* and its relation to pathogenesis. *Curr. Genet.* 44, 1–7.
- Davis, D., Edwards, J. E., Jr., Mitchell, A. P., and Ibrahim, A. S. (2000). *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. *Infect. Immun.* 68, 5953–5959.
- Dosil, M., Giot, L., Davis, C., and Konopka, J. B. (1998). Dominant-negative mutations in the G protein-coupled α -factor receptor map to the extracellular ends of the transmembrane segments. *Mol. Cell. Biol.* 18, 5981–5991.
- Douglas, H. C., and Condrie, F. (1954). The genetic control of galactose utilization in *Saccharomyces*. *J. Bacteriol.* 68, 662–670.
- Dujon, B. (2006). Yeasts illustrate the molecular mechanisms of eukaryotic genome evolution. *Trends Genet.* 22, 375–387.
- Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N., and Wenzel, R. P. (1999). Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin. Infect. Dis.* 29, 239–244.
- Enjalbert, B., and Whiteway, M. (2005). Release from quorum-sensing molecules triggers hyphal formation during *Candida albicans* resumption of growth. *Eukaryot. Cell* 4, 1203–1210.
- Gerami-Nejad, M., Berman, J., and Gale, C. A. (2001). Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in *Candida albicans*. *Yeast* 18, 859–864.
- Hirakawa, H., Inazumi, Y., Senda, Y., Kobayashi, A., Hirata, T., Nishino, K., and Yamaguchi, A. (2006). *N*-acetyl-D-glucosamine induces the expression of multidrug exporter genes, *mdtEF*, via catabolite activation in *Escherichia coli*. *J. Bacteriol.* 188, 5851–5858.
- Holsbeeks, I., Lagatie, O., Van Nuland, A., Van de Velde, S., and Thevelein, J. M. (2004). The eukaryotic plasma membrane as a nutrient-sensing device. *Trends Biochem. Sci.* 29, 556–564.
- Hube, B. (2004). From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. *Curr. Opin. Microbiol.* 7, 336–341.
- James, T. Y. *et al.* (2006). Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* 443, 818–822.
- Kim, J. H., and Johnston, M. (2006). Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 281, 26144–26149.
- Klengel, T. *et al.* (2005). Fungal adenylyl cyclase integrates CO₂ sensing with cAMP signaling and virulence. *Curr. Biol.* 15, 2021–2026.
- Kumamoto, C. A., and Vines, M. D. (2005). Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell. Microbiol.* 7, 1546–1554.
- Kumar, M. J., Jamaluddin, M. S., Natarajan, K., Kaur, D., and Datta, A. (2000). The inducible *N*-acetylglucosamine catabolic pathway gene cluster in *Candida albicans*: discrete *N*-acetylglucosamine-inducible factors interact at the promoter of *NAG1*. *Proc. Natl. Acad. Sci. USA* 97, 14218–14223.
- Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90, 939–949.
- Lorenz, M. C., Bender, J. A., and Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* 3, 1076–1087.
- Maidan, M. M., De Rop, L., Serneels, J., Exler, S., Rupp, S., Tournu, H., Thevelein, J. M., and Van Dijck, P. (2005). The G protein-coupled receptor Gpr1 and the G α protein Gpa2 act through the cAMP-PKA pathway to induce morphogenesis in *Candida albicans*. *Mol. Biol. Cell* 16, 1971–1986.
- Mavor, A. L., Thewes, S., and Hube, B. (2005). Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. *Curr. Drug Targets* 6, 863–874.
- Moriya, H., and Johnston, M. (2004). Glucose sensing and signaling in *Saccharomyces cerevisiae* through the Rgt2 glucose sensor and casein kinase I. *Proc. Natl. Acad. Sci. USA* 101, 1572–1577.
- Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22, 5767–5768.
- Nantel, A. *et al.* (2002). Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* 13, 3452–3465.
- Odds, F. C. (1988). *Candida and Candidosis*. Philadelphia: Bailliere Tindall.
- Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr. (1998). Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* 62, 1–34.
- Rocha, C. R., Schroppel, K., Harscus, D., Marcil, A., Dignard, D., Taylor, B. N., Thomas, D. Y., Whiteway, M., and Leberer, E. (2001). Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell* 12, 3631–3643.
- Schandel, K. A., and Jenness, D. D. (1994). Direct evidence for ligand-induced internalization of the yeast α -factor pheromone receptor. *Mol. Cell. Biol.* 14, 7245–7255.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Sil, A. K., Alam, S., Xin, P., Ma, L., Morgan, M., Lebo, C. M., Woods, M. P., and Hopper, J. E. (1999). The Gal3p-Gal80p-Gal4p transcription switch of yeast: Gal3p destabilizes the Gal80p-Gal4p complex in response to galactose and ATP. *Mol. Cell. Biol.* 19, 7828–7840.
- Simonetti, N., Strippoli, V., and Cassone, A. (1974). Yeast-mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Nature* 250, 344–346.
- Singh, B., and Datta, A. (1979). Regulation of *N*-acetylglucosamine uptake in yeast. *Biochim. Biophys. Acta* 557, 248–258.
- Singh, B. R., and Datta, A. (1978). Glucose repression of the inducible catabolic pathway for *N*-acetylglucosamine in yeast. *Biochem. Biophys. Res. Commun.* 84, 58–64.
- Singh, P., Ghosh, S., and Datta, A. (2001). Attenuation of virulence and changes in morphology in *Candida albicans* by disruption of the *N*-acetylglucosamine catabolic pathway. *Infect. Immun.* 69, 7898–7903.
- Slawson, C., and Hart, G. (2003). Dynamic interplay between O-GlcNAc and O-phosphate: the sweet side of protein regulation. *Curr. Opin. Struct. Biol.* 13, 631–636.
- Sohanpal, B. K., El-Labany, S., Lahooti, M., Plumbridge, J. A., and Blomfield, I. C. (2004). Integrated regulatory responses of fimB to *N*-acetylneuraminic (sialic) acid and GlcNAc in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA* 101, 16322–16327.
- Sonnichsen, B. *et al.* (2005). Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. *Nature* 434, 462–469.
- Spellberg, B. J., Filler, S. G., and Edwards, J. E., Jr. (2006). Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* 42, 244–251.
- Stoldt, V. R., Sonneborn, A., Leuker, C. E., and Ernst, J. F. (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* 16, 1982–1991.
- Sudbery, P., Gow, N., and Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. *Trends Microbiol.* 12, 317–324.
- Sundstrom, P. (2002). Adhesion in *Candida* spp. *Cell. Microbiol.* 4, 461–469.
- Tebarth, B., Doedt, T., Krishnamurthy, S., Weide, M., Monterola, F., Dominguez, A., and Ernst, J. F. (2003). Adaptation of the Efg1p morphogenetic pathway in *Candida albicans* by negative autoregulation and PKA-dependent repression of the *EFG1* gene. *J. Mol. Biol.* 329, 949–962.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Whiteway, M., and Oberholzer, U. (2004). *Candida* morphogenesis and host-pathogen interactions. *Curr. Opin. Microbiol.* 7, 350–357.
- Wilson, R. B., Davis, D., Enloe, B. M., and Mitchell, A. P. (2000). A recyclable *Candida albicans* URA3 cassette for PCR product-directed gene disruptions. *Yeast* 16, 65–70.
- Wilson, R. B., Davis, D., and Mitchell, A. P. (1999). Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J. Bacteriol.* 181, 1868–1874.

- Wu, B., Ottow, K., Poulsen, P., Gaber, R. F., Albers, E., and Kielland-Brandt, M. C. (2006). Competitive intra- and extracellular nutrient sensing by the transporter homologue Ssy1p. *J. Cell Biol.* 173, 327–331.
- Yamada-Okabe, T., Sakamori, Y., Mio, T., and Yamada-Okabe, H. (2001). Identification and characterization of the genes for *N*-acetylglucosamine kinase and *N*-acetylglucosamine-phosphate deacetylase in the pathogenic fungus *Candida albicans*. *Eur. J. Biochem.* 268, 2498–2505.
- Yang, W. H., Kim, J. E., Nam, H. W., Ju, J. W., Kim, H. S., Kim, Y. S., and Cho, J. W. (2006). Modification of p53 with O-linked *N*-acetylglucosamine regulates p53 activity and stability. *Nat. Cell. Biol.* 8, 1074–1083.
- Zachara, N., and Hart, G. (2006). Cell signaling, the essential role of O-GlcNAc! *Biochim. Biophys. Acta* 1761, 599–617.
- Zachara, N. E., and Hart, G. W. (2004). O-GlcNAc modification: a nutritional sensor that modulates proteasome function. *Trends Cell. Biol.* 14, 218–221.