

# *N*-Acetylglucosamine-inducible *CaGAP1* encodes a general amino acid permease which co-ordinates external nitrogen source response and morphogenesis in *Candida albicans*

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Received 31 December 2002

Revised 10 April 2003

Accepted 24 April 2003

*Candida albicans* is able to grow in a variety of reversible morphological forms (yeast, pseudohyphal and hyphal) in response to various environmental signals, noteworthy among them being *N*-acetylglucosamine (GlcNAc). The gene *CaGAP1*, homologous to *GAP1*, which encodes the general amino acid permease from *Saccharomyces cerevisiae*, was isolated on the basis of its induction by GlcNAc through differential screening of a *C. albicans* genomic library. The gene could functionally complement an *S. cerevisiae gap1* mutant by rendering it susceptible to the toxic amino acid analogue mimosine in minimal proline media. As in *S. cerevisiae*, mutation of the *CaGAP1* gene had an effect on citrulline uptake in *C. albicans*. Northern analysis showed that GlcNAc-induced expression of *CaGAP1* was further enhanced in synthetic minimal media supplemented with single amino acids (glutamate, proline and glutamine) or urea (without amino acids) but repressed in minimal ammonium media. Induction of *CaGAP1* expression by GlcNAc was nullified in *C. albicans* deleted for the transcription factor *CPH1* and the hyphal regulator *RAS1*, indicating the involvement of Cph1p-dependent Ras1p signalling in *CaGAP1* expression. A homozygous mutant of this gene showed defective hyphal formation in solid hyphal-inducing media and exhibited less hyphal clumps when induced by GlcNAc. Alteration of morphology and short filamentation under nitrogen-starvation conditions in the heterozygous mutant suggested that *CaGAP1* affects morphogenesis in a dose-dependent manner.

## INTRODUCTION

The ability to use a variety of nitrogen-containing compounds as the sole source of all cellular nitrogen is a predominant feature in yeasts. This ability requires permeases for transport of nitrogenous compounds and enzymes for the generation of ammonia. In response to changes in the environment, there is an increase in the activity of the permeases responsible for uptake of amino acids for use as nitrogen source. This is true for the opportunistic yeast *Candida albicans*, which is the leading aetiological agent of candidiasis, an infection affecting severely immunocompromised individuals (Odds, 1988). Different properties of *C. albicans* have been considered as putative virulence factors, prominent among them being the ability to switch from the yeast to the filamentous form, although both forms of the organism have been found in infected tissue (Cutler, 1991). There are several conditions that promote yeast-to-hyphae morphogenesis *in vitro*,

including growth at an ambient temperature, serum, neutral pH and nutrient starvation (Odds, 1988).

This morphological plasticity reflects the interplay of various signalling pathways which control morphogenesis *in vivo*. In *C. albicans*, Ras1p is an important regulator of hyphal development and likely functions upstream of the cAMP-dependent protein kinase A (PKA) pathway (Feng *et al.*, 1999). In this pathway, two catalytic subunits or isoforms of PKA, Tpk1p and Tpk2p, have differential effects on hyphal morphogenesis under different hyphal-inducing conditions (Bockmuhl *et al.*, 2001). Efg1p, a basic helix-loop-helix (bHLH) protein, plays a major role in hyphal morphogenesis (Leng *et al.*, 2001; Stoldt *et al.*, 1997). *TPK2* overexpression cannot suppress the *efg1/efg1* defect in hyphal development, whereas overexpression of *EFG1* can suppress the filamentation defect in *tpk2/tpk2*, which implies that the function of *EFG1* is downstream of *TPK2* (Bockmuhl *et al.*, 2001; Singh *et al.*, 2001; Sonneborn *et al.*, 2000). Like in *Saccharomyces cerevisiae*, Cph1p/Acpr1p, a homologue of Ste12p (Liu *et al.*, 1994; Singh *et al.*, 1994, 1997), and a MAP kinase cascade that includes Cst20p (p<sup>21</sup>-activated kinase; PAK) (Leberer *et al.*, 1996, 1997), Hst7p (MAP kinase kinase; MEK) (Leberer *et al.*, 1996) and

Abbreviation: PRE, pheromone responsive element.

The EMBL accession number for the sequence reported in this paper is AF467941.

Cek1p (MAPK) (Csank *et al.*, 1998) are also involved in filamentation in *C. albicans*. Most importantly, GlcNAc has a dual role to play in that it not only induces the synthesis of its catabolic enzymes, a kinase (Hxk1p), a deacetylase (Dac1p) and a deaminase (Nag1p) (Kumar *et al.*, 2000), but also regulates GlcNAc-induced transition from a yeast to hyphal form (Singh *et al.*, 2001). Filamentation regulated by the Nag regulon (*HXK1/DAC1/NAG1*) is independent of Tpk2p and the Cph1p/Acpr1p-regulated MAP kinase pathway but is dependent on the morphological regulator Efg1p (S. Ghosh and others, unpublished data).

In order to identify and characterize the genes that could be involved in the regulation of morphogenesis and virulence induced by GlcNAc, we performed differential screening of a *C. albicans* genomic library to identify the genes that are regulated specifically by GlcNAc. Here we report the identification and characterization of the GlcNAc-inducible gene *CaGAP1*, which is homologous to *GAP1*, which encodes a general amino acid permease of *S. cerevisiae*. In yeast, Gap1p is a low-affinity permease with low specificity, which is highly regulated in response to the available nitrogen source (Sophianopoulon & Diallyonias, 1995). In the presence of ammonia or glutamine, the amino acid uptake is low, whereas in media containing a poor nitrogen source, e.g. proline, the amino acid uptake is high (Blinder *et al.*, 1996; Courchesne *et al.*, 1983). In *S. cerevisiae*, at least five proteins (Ure2p, Dal80p, Gln3p, Nil1p and Nil2p) function co-ordinately to control the transcription of *GAP1* (Blinder *et al.*, 1996; Cunningham *et al.*, 1993; Rowen *et al.*, 1997; Stanbrough *et al.*, 1995). The nitrogen-dependent regulation of *GAP1* is complex, occurring not only at the level of *GAP1* transcription but also through Gap1p sorting and degradation by ubiquitin-triggered internalization (Springael *et al.*, 1998).

In this report, complementation studies by expressing *CaGAP1* in a *gap1* mutant of *S. cerevisiae* showed the functional similarity of CaGap1p with the general amino acid permease (Gap1p) of *S. cerevisiae*. We observed certain differential expression of *CaGAP1* in various nitrogen sources as well as in mutants defective in morphogenesis and virulence. We also report some conditions where filamentation and morphogenesis were altered in heterozygous and homozygous disruptants of *CaGAP1*.

## METHODS

**Strains and media.** All strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cultures were grown at 37 °C in either TB (1.2% Bacto tryptone, 2.4% yeast extract, 0.4% glycerol) or LB (1% tryptone, 5% yeast extract, 5% NaCl, 1 mM NaOH). *C. albicans* and *S. cerevisiae* strains were cultured at 30 °C in either YPD (1% yeast extract, 2% Bacto peptone, 2% glucose) or SD (0.67% yeast nitrogen base without amino acids and 2% glucose) medium. Minimal-Proline medium (MIN-Proline/SPD) contained 0.67% yeast nitrogen base without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and without amino acids, 2% glucose and 1 g proline l<sup>-1</sup> (added after autoclaving). This medium was used to select and score the mutation, which conferred resistance to the amino acid analogue mimosine

(75 µg ml<sup>-1</sup>). Min-Glutamate (SED) and Min-Ammonium (SAD) media contained 1 g glutamate l<sup>-1</sup> or 2 g ammonia l<sup>-1</sup>. All solid media contained 2 g agar l<sup>-1</sup>. GPK (0.5% glucose, 0.5% peptone and 0.3% KH<sub>2</sub>PO<sub>4</sub>) and NPK (0.5% GlcNAc, 0.5% peptone and 0.3% KH<sub>2</sub>PO<sub>4</sub>) were used for GlcNAc induction studies in *C. albicans*. For analysing the induction effect of alternative nitrogen sources, glutamate, proline, ammonia, urea and glutamine were used along with 0.67% yeast nitrogen base without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and without amino acids, and 2% GlcNAc. The respective media were named SEN, SPN, SAN, SUN and SGN. GlcNAc and the other nitrogen sources glutamate (1 g l<sup>-1</sup>), proline (1 g l<sup>-1</sup>), ammonia (2 g l<sup>-1</sup>), urea (2 g l<sup>-1</sup>) and glutamine (1 g l<sup>-1</sup>) were filter-sterilized and added after autoclaving. The induction effect of a single amino acid or urea was shown here with respect to synthetic complete (SN) GlcNAc medium. In the same way, SED, SPD, SAD, SUD, SGD and SD were prepared with 2% glucose in place of GlcNAc. SC medium contains 0.67% yeast nitrogen base [with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and amino acids] and 2% glucose.

**Isolation of *CaGAP1*.** The *CaGAP1* gene was isolated by differential screening of a *C. albicans* genomic library in Yep13 with cDNA probes synthesized from poly(A<sup>+</sup>) RNA of glucose-grown (uninduced) and GlcNAc-grown (induced) cells (Okayama *et al.*, 1987). The clone was subsequently sequenced. The sequence data were matched with the *C. albicans* Genome Sequencing Project, Stanford, followed by ORF analysis through ORF Finder, NCBI.

**Construction of a *CaGAP1* expression vector plasmid in *S. cerevisiae*.** The *CaGAP1* coding region was PCR-amplified from genomic DNA of *C. albicans* SC5314 using the oligonucleotides 5'-TGATCCTTTAATCTTGGAGAAGG-3' and 5'-TGTTCAACCTGTCAAAGTCC-3' as primers. The 2.2 kb PCR fragment was cloned into the pGEMT-Easy vector followed by transformation into *E. coli* strain DH5 $\alpha$ , as per the manufacturer's instructions (Promega), generating pGPFORF. A 2224 bp gel-purified *NotI* fragment containing the *CaGAP1* ORF and downstream portion of the ORF was subcloned into pFL61, a yeast expression vector, under the PGK promoter, generating pFLGP31.

**Complementation study of *CaGAP1* in *S. cerevisiae*.** Transformation of *S. cerevisiae* was carried out by the lithium acetate method as described by Gietz *et al.* (1992). Five micrograms of plasmid pFLPF31 along with 50 µg denatured calf thymus DNA was transformed into the *S. cerevisiae gap1* strain MS143. The transformation mix was plated on SD-URA medium using *URA3* as a selection marker. The MS143 ( $\Delta gap1$ ) strain was plated as a control. The transformants were replica-plated on SD medium containing a suitable amount of supplements without uridine. Ura-positive transformants ( $\Delta gap1::CaGAP1$ ) were tested on minimal proline plates containing 20 µg uridine ml<sup>-1</sup> and 75 µg mimosine ml<sup>-1</sup>.

**Assay of amino acid uptake.** *S. cerevisiae* and *C. albicans* strains to be assayed were cultured in SD medium to OD<sub>600</sub> ~2.0. Cells were collected by filtration on 0.45 µm nitrocellulose filters (Sartorius) and resuspended in SPD medium. [<sup>14</sup>C]Citrulline was added to exponentially growing cultures. Samples of 0.5 ml were removed periodically for 2.5 min, rapidly collected by filtration through a glass fibre filter (Whatman) and washed with chilled water. Filters were dried under a heat lamp and placed in 5 ml toluene-based liquid scintillation cocktail. The counts were taken in a Wallac DSA-based liquid scintillation counter. The specific activity of [<sup>14</sup>C]citrulline used was 2.1 GBq mmol<sup>-1</sup>. Labelled citrulline was obtained from Perkin Elmer Life Sciences.

**GlcNAc induction studies of *CaGAP1*.** *C. albicans* SC5314 cells were precultured in GPK medium and resuspended in 100× volume of fresh GPK. Cultures were grown to OD<sub>600</sub> ~2.0. Harvested cells were washed twice with 0.3% KH<sub>2</sub>PO<sub>4</sub>, resuspended in equal

**Table 1.** Strains and plasmids used in this study

Strain/plasmid	Genotype/description	Source/reference
<b>Strains</b>		
<i>E. coli</i>		
DH5 $\alpha$	F'/endA1 hsd R17 (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>+</sup> ) glnV44 thi-4 recA1 gyrA (Nal <sup>r</sup> ) relA1 $\Delta$ (lacI ZYA-argF) U169 deoR [ $\phi$ 80dlac $\Delta$ (lacZ)M15]	
<i>S. cerevisiae</i>		
MS138	MATa ura3-52 leu2-3 GAP1	M. C. Brandriss, NJMS
MS143	MATa ura3-52 leu2-3 $\Delta$ gap1::LEU2	M. C. Brandriss, NJMS
MSPF31	MATa ura3-52 leu2-3 $\Delta$ gap1::LEU2 [CaGAP1]	This work
<i>C. albicans</i>		
SC5314 (wild-type)	URA3/URA3	
CAF-3-1(wild-type)	$\Delta$ ura3::imm434/ $\Delta$ ura3::imm434	W. A. Fonzi, Georgetown University, Washington, DC, USA
GP-5	As CAF3-1, but CaGAP1/ $\Delta$ Cagap1::hisG-URA3-hisG	This work
GP-57	As CAF3-1, but CaGAP1/ $\Delta$ Cagap1::hisG	This work
GP-573	As CAF3-1, but $\Delta$ Cagap1::hisG-URA3-hisG/ $\Delta$ Cagap1::hisG	This work
GP-5731	As CAF3-1, but $\Delta$ Cagap1::hisG/ $\Delta$ Cagap1::hisG	This work
GP-57315	As CAF3-1, but $\Delta$ Cagap1::hisG/ $\Delta$ Cagap1::URA3	This work
N-2-1-6	As CAF3-1, but $\Delta$ dac1-proA $\Delta$ nag1 $\Delta$ hcx1::hisG/ $\Delta$ dac1-proA $\Delta$ nag1 $\Delta$ hcx1::hisG-URA3-hisG	Laboratory strain
N-2-1-6-1+p33	As CAF3-1, but $\Delta$ dac1-proA $\Delta$ nag1 $\Delta$ hcx1::hisG/DAC1 NAG1 $\Delta$ hcx1::hisG-URA3-hisG	Laboratory strain
CAN52	$\Delta$ ras1::hisG/ $\Delta$ ras1::hph $\Delta$ ura3::imm434/ $\Delta$ ura3::imm434	Feng <i>et al.</i> (1999)
HLC67	$\Delta$ efg1::hisG/ $\Delta$ efg1::hisG $\Delta$ ura3::imm434/ $\Delta$ ura3::imm434	Bockmuhl <i>et al.</i> (2001)
AS1	$\Delta$ tpk2::hisG/ $\Delta$ tpk2::hisG $\Delta$ ura3::imm434/ $\Delta$ ura3::imm434	Bockmuhl <i>et al.</i> (2001)
A-11-1-1-4 (cph1 <sup>-</sup> )	$\Delta$ acpr1::hisG/ $\Delta$ acpr1::hisG $\Delta$ ura3::imm434/ $\Delta$ ura3::imm434	Laboratory strain
<b>Plasmids</b>		
pGPORF	CaGAP1 ORF cloned in pGEM-T Easy vector	This work
pFL61	URA marked ScARS vector plasmid	ATCC
pFLGP31	CaGAP1 ORF subcloned into pFL61 under PGK promoter	This work
pCaGAP1	3.5 kb CaGAP1 fragment cloned in pGEM-T Easy vector	This work
pGP1	Carrying $\Delta$ Cagap1::hisG-URA3-hisG disruption fragment	This work
pGP2	Carrying CaGAP1::URA3 reconstruction fragment	This work
pCUB6	Carrying hisG-URA3-hisG disruption cassette	W. A. Fonzi, Georgetown University, Washington, DC, USA
pUC19-CUB	hisG-URA3-hisG disruption cassette cloned in pUC19	This work

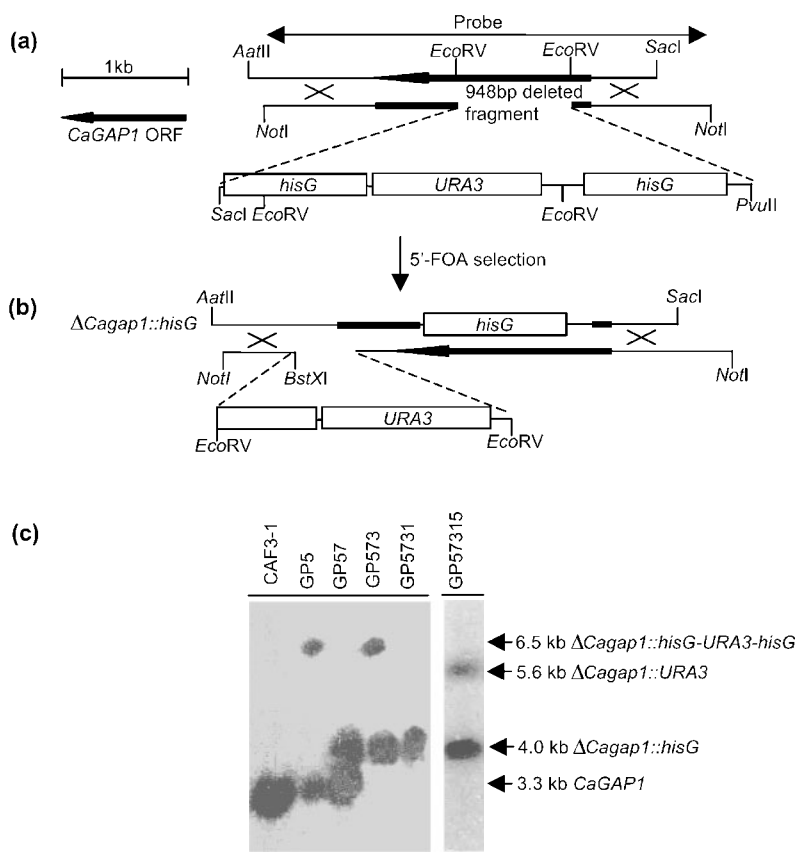
volume of NPK, and incubated at 30 °C. The treated cells were harvested at different time points of growth as described in Results and frozen at -20 °C until use. Control cells were resuspended in GPK instead of NPK.

To see the effect of GlcNAc induction in different nitrogen sources, strain SC5314 was precultured in SC, washed once with water, resuspended in SN, SEN, SPN, SAN, SUN and SGN with 2 % GlcNAc and grown for 2 h at 30 °C. The treated cells were harvested and frozen at -20 °C until use. Control cells were resuspended in different media, SD, SED, SPD, SAD, SUD and SGD, with 2 % glucose as a carbon source. For studying the effect of GlcNAc induction in different mutants of *C. albicans* strains, N-2-1-6, N-2-1-6+p33, A-11-1-1-4, CAN52, AS1 and HLC67 were grown similarly in SN medium with 2 % GlcNAc for 2 h at 30 °C and control cells were cultured in SC with 2 % glucose.

**RNA extraction and Northern analysis.** Total RNA was extracted from frozen cells (Ausubel *et al.*, 1994). Then 1.5 % formaldehyde agarose gel electrophoresis was carried out with 40  $\mu$ g RNA per lane,

and subsequent Northern blot analysis was performed as described by Ausubel *et al.* (1994) with a <sup>32</sup>P-labelled 938 bp EcoRV fragment of CaGAP1 (see Fig. 5a), excised from pCaGAP1.

**Construction of mutant strains of *C. albicans*.** Disruption of the general amino acid permease gene was performed according to the URA-blaster protocol (Fonzi & Irwin, 1993). The entire 3512 bp CaGAP1 fragment was PCR-amplified from genomic DNA of *C. albicans* SC5314 using the oligonucleotides 5'-CATTACC-TGGTGCCACTCC-3' and 5'-GGTTTCGAATCAGTCGATGG-3' as primers and cloned in the pGEMT-Easy vector followed by transformation into DH5 $\alpha$  to generate pCaGAP1. To obtain  $\Delta$ Cagap1 mutants, plasmid pGP1 was constructed by replacing a 948 bp EcoRV-EcoRV fragment of pCaGAP1 containing the CaGAP1 ORF with a 4176 bp blunt-ended SacI-PvuII fragment of vector pUC19-CUB containing the hisG-URA3-hisG cassette. To obtain pUC19-CUB, the 4 kb BamHI-BglII hisG-URA3-hisG cassette from pCUB6 was integrated into the BamHI site of plasmid pUC19. CAF-3-1 was transformed by the lithium acetate method (Gietz *et al.*, 1992) with a 6688 bp NotI fragment derived from the targeting construct pGP1



**Fig. 1.** Schematic representation of the construction of the cassette used to disrupt *CaGAP1* (a) and the cassette *CaGAP1-URA* used to reintroduce one wild-type *CaGAP1* allele (b). (c) Corresponding Southern blot analysis of strains CAF3-1 (wild-type +/+ *Ura*<sup>+</sup>), GP5 (+/*ΔCagap1 Ura*<sup>+</sup>), GP57 (+/*ΔCagap1 Ura*<sup>+</sup>), GP573 (*ΔCagap1/ΔCagap1 Ura*<sup>+</sup>), GP5731 (*ΔCagap1/ΔCagap1 Ura*<sup>-</sup>) and revertant strain GP57315 (*ΔCagap1/ΔCagap1 + CaGAP1 Ura*<sup>+</sup>) obtained during the disruption process. Genomic DNA from these strains was *AatII/SacI*-digested and hybridized with a 3.5 kb *NotI* fragment of plasmid pCaGAP1. The exact size and genotype of the expected hybridizing DNA fragment are indicated on the right.

(Fig. 1a). Transformants were selected on synthetic minimal media (SD) to obtain *Ura*<sup>+</sup> transformants. After confirmation of disruption by Southern analysis, *Ura*<sup>+</sup> transformants (GP5) were screened for *Ura*-cured segregants on SD minimal medium containing 1 mg 5'-fluoroorotic acid ml<sup>-1</sup> and 25 μg uridine ml<sup>-1</sup>. One *Ura*-cured transformant (GP57) was then used to delete the second allele of *CaGAP1* using a similar process to generate the homologous *gap1/gap1* mutants GP573 (*Ura*<sup>+</sup>) and GP5731 (*Ura*<sup>-</sup>).

**Construction of *CaGAP1* revertant strain GP57315 in *C. albicans*.** In order to obtain a reconstituted strain with one *CaGAP1* allele, we constructed the plasmid pGP2 where a 2.3 kb *EcoRV-EcoRV* fragment from pUC19-CUB containing *URA3* was introduced into the *BstXI* site of pCaGAP1 located downstream of the *CaGAP1* ORF. The homozygous mutant GP5731 (*Ura*<sup>-</sup>) was then transformed with a 5.8 kb *NotI* fragment derived from pGP2 (Fig. 1b). Transformants were selected on SD minimal medium to obtain a *Ura*<sup>+</sup> strain, which was confirmed by Southern analysis.

**Southern analysis.** For screening of mutants and revertant strains, 5 μg genomic DNA from each transformant and parent strain was digested with *AatII* and *SacI*, electrophoresed and transferred (Sambrook *et al.*, 1989) to Genescreen Plus membrane (NEN Research Products). The blots were hybridized with a <sup>32</sup>P-labelled 3.5 kb *NotI-NotI* fragment from pCaGAP1 (Fig. 1c).

**Induction of filamentation by serum and GlcNAc.** *Candida* cells were grown to the exponential growth phase in YPD, washed twice with sterile water and shaken for 10 h in water at 30 °C and 100 r.p.m. (Sonneborn *et al.*, 2000). Cells (OD<sub>600</sub> 0.5) were then induced for germ tube formation with 2.5 mM GlcNAc in salt base

containing 0.45% NaCl and 0.335% YNB without amino acids at 37 °C for 4 h or with bovine calf serum (Sigma) in YPD at 37 °C for 2 h.

**Morphogenesis studies on solid media.** *Candida* strains were grown in SD at 30 °C, counted using a haemocytometer, and plated at a concentration of 80–100 cells per Spider (1% nutrient broth, 1% mannitol, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 2% Bacto agar) or SLAD (0.17% YNB without amino acids and ammonium sulphate, 2% glucose, 50 μM ammonium sulphate, 2% Bacto agar) plate. Plates were incubated at both 30 and 37 °C for 7–10 days.

**Determination of virulence.** Female Swiss mice, 5–6 weeks old, were intravenously injected with 10<sup>6</sup> cells of wild-type (SC5314), heterozygous *Cagap1* mutant (GP5), homozygous mutant (GP573) and revertant (GP57315) strains of *C. albicans*. The number of surviving mice was scored.

## RESULTS

### Sequence analysis of the *CaGAP1* gene in *C. albicans*

Sequencing of the *C. albicans CaGAP1* gene followed by a BLAST search revealed that it is homologous to *GAP1*, which encodes the general amino acid permease from *S. cerevisiae* (Jauniaux & Grenson, 1990). The sequence was submitted to the EMBL database and assigned accession number AF467941. The sequence contained a single ORF of 1746 nucleotides, which encodes a predicted protein of 582 amino acids with an estimated *M<sub>r</sub>* of 63 950. Several

putative TATA box sequences along with a global regulator, Gcn4p, and AP-1 binding site appear at positions -286 and -152, respectively, upstream from the initiation codon ATG. Interestingly, there is one 5'-GAATAG-3' sequence (at the -646 position), and several TTGTT/TGGTT sequences were found upstream of the *CaGAP1* promoter. GAATAG/GATA-type sequences which are the binding target of the transcription factor Gln3p were also found in the *GAP1* promoter of *S. cerevisiae* (Miller & Magasanik, 1991). TTGGT or TTGTT plays an auxiliary role in activation of nitrogen-regulated genes by Gln3p (Stanbrough *et al.*, 1995). Another transcription factor, Cph1p/Acrp1 of *C. albicans*, a homologue of Ste12p of *S. cerevisiae*, binds to a heptamer sequence, TGAAACA, referred to as a pheromone responsive element (PRE). This sequence is also present at the -989 position of the *CaGAP1* promoter (Fig. 2). Comparison of the predicted CaGap1p amino acid sequence with the *S. cerevisiae* genome database (<http://genome-www.stanford.edu/Saccharomyces/>) using the CLUSTALW program (<http://www.ebi.ac.uk/>) revealed that *CaGAP1* bears a marked resemblance to some previously sequenced yeast permease genes, such as *HIP1* (histidine permease), *TAT2* (tryptophan permease), *AGP1* (arginine/glutamate permease), etc., with an overall sequence similarity of 40–50%. See the supplementary figure at <http://mic.sgmjournals.org>.

### Hydropathy profile

The protein product (AAL76065.1) that was deduced from the *CaGAP1* gene sequence is considerably hydrophobic,

containing 46% non-polar residues. A hydropathy profile generated with the Kyte & Doolittle (1982) algorithm showed that there are 10–12 transmembrane regions within the protein (Fig. 3a). Hydrophobic segments of at least 20 amino acids were revealed with a mean hydropathy value lower than 1.3, suggesting the formation of membrane-spanning  $\alpha$  helices by these segments (Lodish, 1988). These transmembrane regions are interconnected with hydrophilic regions that frequently contain a cluster of positively or negatively charged amino acids (Fig. 3b). The N-terminus of the CaGap1p polypeptide is hydrophilic, like those of many integral membrane proteins, and does not present the feature of a cleavable signal peptide sequence predicted by the ExpASY (Expert Protein Analysis System) proteomics ([http://www.expasy.ch/proteomics\\_def.html](http://www.expasy.ch/proteomics_def.html)) server of the Swiss Institute of Bioinformatics (<http://www.isb-sib.ch/>).

### *C. albicans* CaGAP1 is a functional homologue of *S. cerevisiae* GAP1

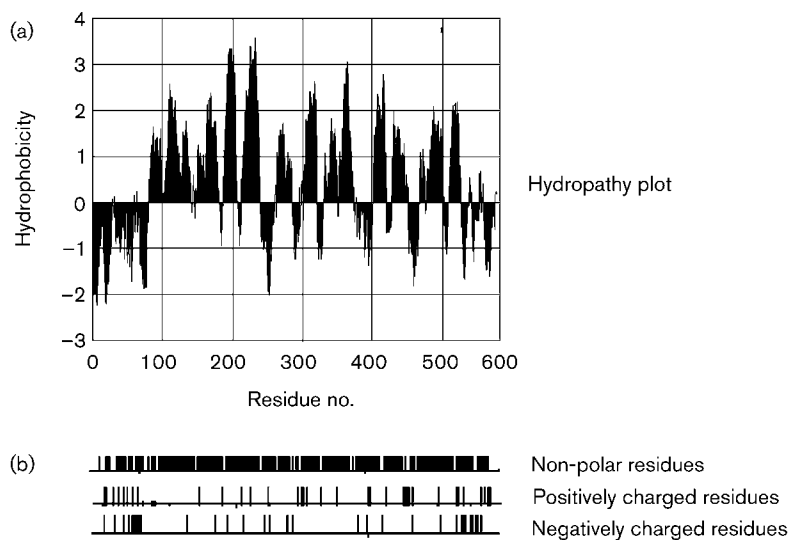
The general amino acid permease Gap1p of *S. cerevisiae* is responsible for the transport of all the natural amino acids and related compounds, such as ornithine and citrulline, and several D-amino acids and toxic amino acid analogues such as mimosine (Rytka, 1975). Therefore, a *gap1* mutant of *S. cerevisiae* is able to survive in the presence of D-amino acids and mimosine (McCusker *et al.*, 1990). In the course of this study, we found that the haploid ancestor strain (MS138) of the *gap1* mutant (MS143) of *S. cerevisiae* and the transformants ( $\Delta gap1::CaGAP1$ ) failed to grow on

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-1078 TAAATCGTTTCTGAACTTCCAGTTAAAACTAACGGAGACTCATTACAAGTCAAACAACCTTPTAAAGATA -1004
      ATTTTACCAAGACTTTGAAAGCTCAATTTTTGATTGCTCTGAGTAAGTGTTCAGTTTGTGAAATATTTCTAT
-1003 TTTCTGGATTTGAAACGACAAATTAATGAGACCAACCAAAATTTGTTAAAGAAGAGTTGTAGAGATTAATAATTCAT -929
      AAGACCTAARCTTTGCTCTTAATTAACCTCTGCTTGGGTTAACAATTTCTCTCAACAATCTCTAATTTTTAAGTA
      PRE binding site
-928 TTTTGAACCAACAGAGCAATTTAACCTCCAATCCAAGACTTGGGTTTCGAATCAGTCGATGGTTCTAGAAAGCA -854
      AAAACCTGCTGCTTAAATTTGAGAGTTTAGGTTCTGAAACCAAGCTTAGTCAGCTACCAAGATCTTTCTGT
-853 GGTTCCTGGAACAAACAACACTACGTTCTGGAATATTTGGGAAGGAGCGCAATTTATTTGAGACTTCAACAACA -779
      CCAAGACCTTGTTTGTTTGTGCAAGACTTTAATAACCTTCTCGCGGTTAATAAATCTGAAAGTGTGTTG
-778 AATGCCAGTATACTTACAGAAAAATAAATCAACAGTCCATAGAAATACAACAATTTGACAACCTCAAATCGAAG -704
      TTACGGCTATATGAAATGCTTTTATTTAGTTGTCAGGTACTTTATGTTGTTTAACTGTTGAGTTTAGCTTC
-703 GACAAAATAAGACATACAAGAAATCAAACAATTTTAAAAATTTTGGGTTCCCTCAATAGTTGGAATCGAAGCAT -629
      CTGTTTATTTCTGTATGTTCTTTAGTTTGGTTAAAAATTTAATAACCAGGATCTTATCAACCTTAGCTTCGTA
      GATA binding site
-628 CTTATAAGTACTTTCAACTGAAATCCGATAATGGAAGCCAAACCAAAACCAAGACCAATPAAACGGAAAAAACA -554
      GGATATTTCAATGAAAGTTGACTTTTAGGCTATACCTTTTGGTTTGGGTTCTGGTTATTTTGGCTTTTTTTG
-553 AAAGCTAGAGCTCTTGGCTTTTCGGGAATGTGAAAGCGATTTTAAAGTTTCTGCTTTGCTCACATGACGGAATC -479
      TTTGATCTCGAGGAGCGGAAAGCCCTTACACTTTCGCTAAAAATTCAAAGACGGAAACGAGTACTGCTTGTAG
-478 TAAATTTGCTGTGTCAGATTTAATTTCTTTTTTTTTTTTTTTTTCGCTTCAACGGCAAAATCAACCTTAAACGCA -404
      ATTTTAAACACACAAAGCTCAAAATTAAGAAAAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATAA
-403 AGGCATTTTGTACTCGGCAAGAGATGACCAAGTTTCTCCATAACTCTCTGCTTTATTTGGTTTACAAAGAAA -329
      TCCGTAATAAATCAATGAGCCCTCTCTACTGGTTCAAACAGGTAATGAGAGAACGAAATACCAACATGTTCTTT
-328 AGTTTTTATGTTCAACTGGTCAAAGTCCCTACAAGATTTATGACTCAATTTATCAAGTTGCTTTTGGAAACTTAT -254
      TCAAAAATACAAGTTGACCCAGTTTCCAGGAGTGTCTAATTAAGTAAAGTAAAGTAAAGTAAAGTAAAGTAAAGT
      GCN4 binding site
-253 AAAAATGCTGGGTTTCTTTTATGACATTTTAAAAAGCAAAAATAAATAAAGTTCCTTTTTTTCTTTAGCTTT -179
      TTTTTAACGACCCAAAGGAAAAATACGATACATTTTTTCTTTTTTTTATTTTCAAGAAAAAGAAAGAAATCGAA
-178 TGACAATATGATATATGTTGGTGTGAGTAAGCAATGACTTCTTTATATATATAAATAACTACTCATCATCAGCATCTT -104
      ACTGTATATATACACCACTCACTTCTGTTACTGAAAGAAATATATATATAATTTGATGAGTAGTAGTCGTAGAA
      AP-1 binding site
-103 TCTTTGTAACATTTTTCAAAAATAGAACTTATTTGTAAGTTTGTAGTTTCTTTTCTTTTATCTTACATCAAGAC -29
      AGAAACATTTGTAAGAGTTTATTTATCTTTAGAAATAAACAATCAACAATAAAGAAAGAAATAGAAATGATGTTCTG
-28 TCTTCTTTGCTCAATACCAATCATCATCATGCTTACATAAAAAAGAACTAACGATACATTTGTTCAATTAATATAG 46
      AGAAGAAACGAGTTATGTTAGTAGTAGTACGATGATTTTTCTTTTGTATGCTATGTAACAACAGTTAATTTATC
      M L H K K

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**Fig. 2.** *CaGAP1* promoter sequence. Nucleotides are numbered on the right from the ATG initiation codon of the *CaGAP1* ORF (position +1). The GAATAG, PRE-binding, TTGTT and TTGGT sites are boxed. The Gcn4p-binding and the AP-1-binding sites are underlined.



**Fig. 3.** Hydropathy plot. (a) Hydrophobicity and hydrophilicity values (above and below the horizontal line, respectively) deduced with the algorithm of Kyte & Doolittle (1982), using a 20-amino-acid window. (b) Distribution of non-polar and positively and negatively charged residues.

minimal proline media (SPD) containing mimosine whereas the *gap1* mutant MS143 was not sensitive to mimosine (Fig. 4a, c), indicating the functional similarity of CaGap1p with Gap1p of *S. cerevisiae*. The failure of the transformants ( $\Delta gap1::CaGAP1$ ) to grow on minimal proline medium containing mimosine is probably the result of mimosine uptake by CaGap1p. The growth rate of a *Cagap1* null mutant of *C. albicans* (GP573) is higher in comparison to the wild-type strain SC5314 and a revertant strain, GP57315, in glucose-containing minimal proline media, SPD (Fig. 4e), and GlcNAc-containing minimal proline media, SPN (Fig. 4f), when mimosine was added. However, the effect of mimosine in *C. albicans* persists only up to a maximum of 10–15 h, as a result of which we found no significant difference in growth on solid plates after 2 days (Fig. 4g, h). This may be due to a higher growth rate of *C. albicans* as compared to *S. cerevisiae*.

To explore whether CaGap1p of *C. albicans* allows uptake of amino acids and related compounds, a citrulline uptake assay was performed in minimal proline medium (SPD) to demonstrate the general amino acid permease activity in *S. cerevisiae* as well as in *C. albicans*. In the *Cagap1* null mutant (GP573), the citrulline uptake rate was two times lower than in the wild-type strain (SC5314) and a revertant strain (GP57315) in *C. albicans* (data not shown). Interestingly, the citrulline uptake of transformants ( $\Delta gap1::CaGAP1$ ) was increased 2.5-fold over that of the *gap1* mutant of *S. cerevisiae*. That indicates the functional similarity of the general amino acid permease of the two micro-organisms.

#### Effect of nitrogen source on the amino acid analogue resistant phenotypes

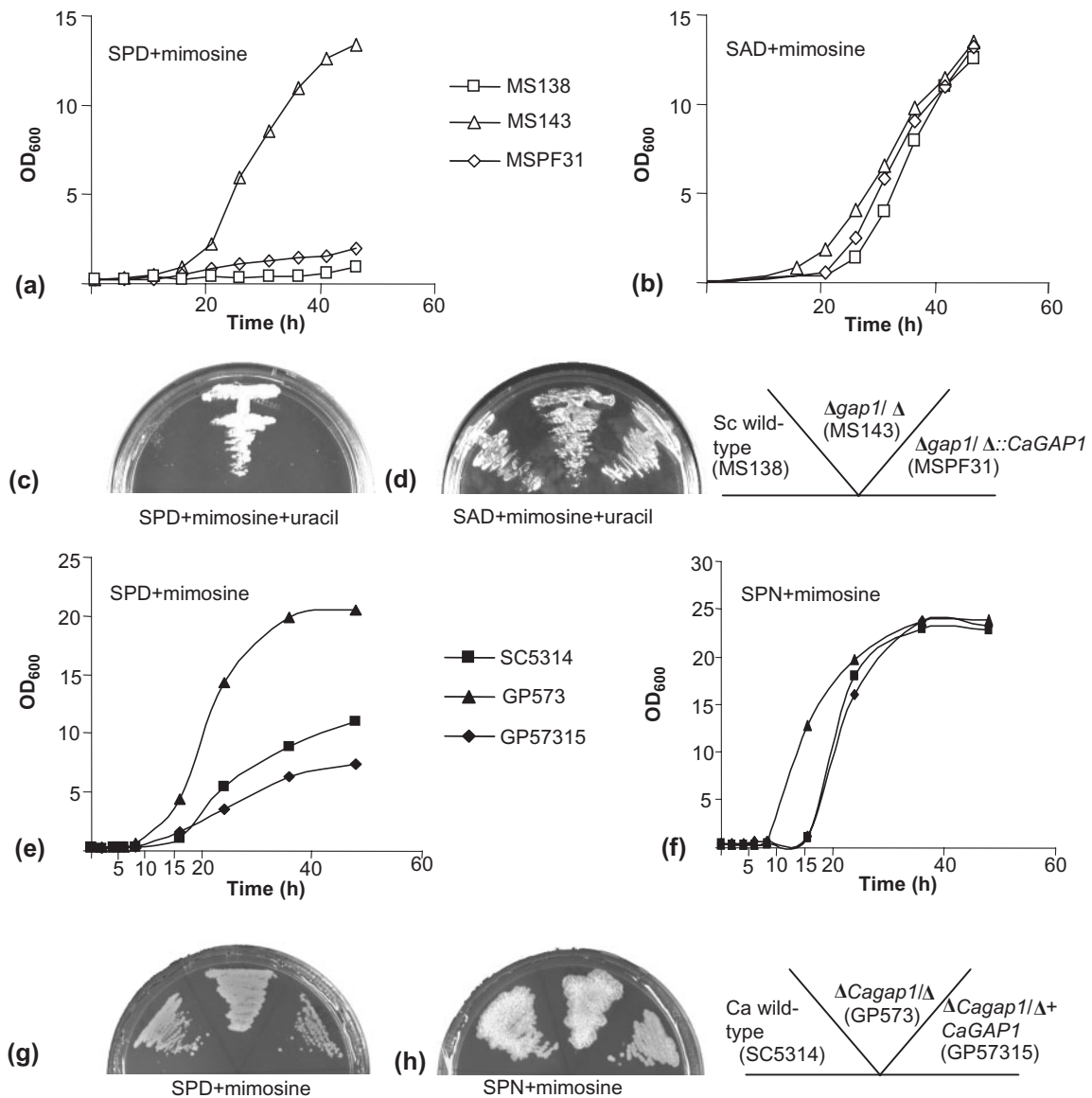
Amino acids are transported into *S. cerevisiae* by both specific and non-specific transport systems. The general amino acid permease system is strongly repressed when growth medium contains  $(NH_4)_2SO_4$  and glutamate (Springael & Andre, 1998). To investigate such an effect

on the regulation of CaGap1p we did growth kinetics as well as replica plating of wild-type strain MS138, the mutant MS143 ( $\Delta gap1$ ) and transformants ( $\Delta gap1::CaGAP1$ ) of *S. cerevisiae* on media containing ammonia (SAD) and glutamate (SED) as nitrogen source in the presence of mimosine. Interestingly, the wild-type strain and the transformants were found to be resistant to mimosine in SAD (Fig. 4b, d) and SED (data not shown). These results suggested that in ammonia- and glutamate-containing medium, mimosine uptake is lowered due to the inactive general amino acid permease system. Moreover, we observed a similar effect when we did growth kinetics as well as 2 days incubation on solid plates of the *C. albicans* wild-type strain SC5314 and the null mutant GP573 in ammonia- and glutamate-containing media using both glucose and GlcNAc as a carbon source (data not shown).

#### Effect of different nitrogen sources on GlcNAc induction of CaGAP1

The *CaGAP1* gene was isolated as a result of its differential expression in glucose- and GlcNAc-grown cells. Northern analysis was used to investigate the expression of *CaGAP1* in glucose-grown and GlcNAc-grown cultures at various intervals. A significant induction was observed in GlcNAc-grown cells at 2 h growth (Fig. 5b).

Northern blot analysis was also used to investigate the effect of different nitrogen sources upon GlcNAc induction of *CaGAP1* (Fig. 5c). The intensity of the individual bands was quantified by densitometry of the autoradiogram, and the fold induction has been represented graphically in Fig. 5(d). It was observed that in SEN (glutamate), SPN (proline), SUN (urea) or SGN (glutamine) media, the level of *CaGAP1* mRNA was about 1.4-fold higher than that of control cells grown in only GlcNAc-containing medium (SN), whereas the *CaGAP1* mRNA level was very low in ammonium-containing SAN medium. There was no change in the level of expression in histidine-containing SHN medium (data not shown). The same experiment was



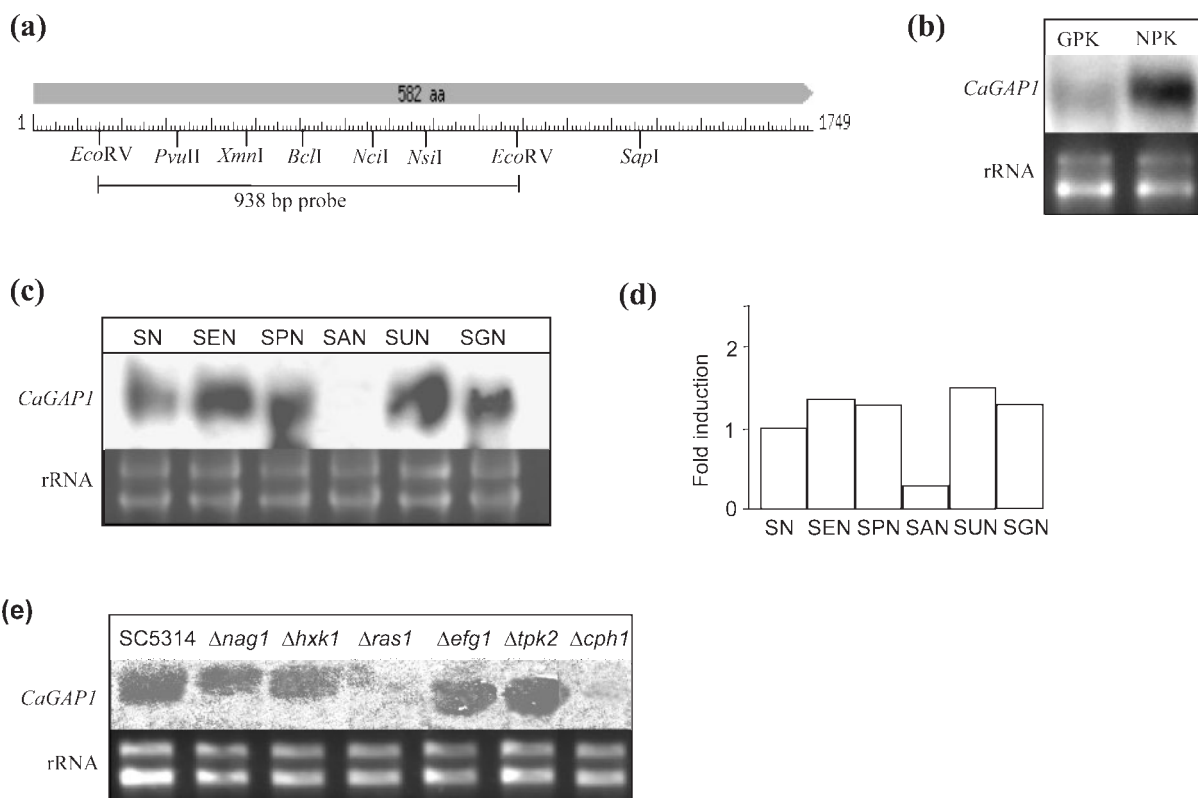
**Fig. 4.** Phenotype of a general amino acid mutant strain of *S. cerevisiae* and *C. albicans*. *S. cerevisiae* strains MS138 (wild-type), MS143 ( $\Delta gap1/\Delta$ ) and MSPF31 ( $\Delta gap1/\Delta::CaGAP1$ ) were incubated in liquid glucose-containing minimal proline medium, SPD (a), and glucose-containing minimal ammonium medium, SAD (b), at 30 °C for the indicated time period. (c, d) *S. cerevisiae* strains MS138, MS143 and MSPF31 grown on solid SPD (c) and SAD (d) at 30 °C for 2 days. (e, f) *C. albicans* strains SC5314 (wild-type), GP573 ( $\Delta Cagap1/\Delta$ ) and GP57315 ( $\Delta Cagap1/\Delta+CaGAP1$ ) incubated in SPD (e) and GlcNAc-containing minimal proline medium, SPN (f), at 30 °C for the indicated time period. (g, h) *C. albicans* strains SC5314, GP573 and GP57315 grown on solid SPD (g) and SPN (h) at 30 °C for 2 days.

carried out using SED, SPD, SAD, SUD, SGD and SHD media where glucose was supplied as carbon source, but no induction or repression was observed (data not shown).

#### Expression of CaGAP1 is regulated by Cph1p-mediated Ras1p signalling but is independent of Efg1p

To investigate the effect of different mutations on the expression of GlcNAc-inducible CaGAP1, strains N-2-1-6

( $\Delta dac1\Delta nag1\Delta hxc1/\Delta dac1\Delta nag1\Delta hxc1$ ), N-2-1-6 + p33 ( $\Delta dac1\Delta nag1\Delta hxc1/DAC1NAG1\Delta hxc1$ ), A-11-1-1-4 ( $\Delta acpr1/\Delta acpr1$ ), CAN52 ( $\Delta ras1/\Delta ras1$ ), HLC67 ( $\Delta efg1/\Delta efg1$ ) and AS1 ( $\Delta tpk2/\Delta tpk2$ ) were used. Northern blots showed that transcript levels of CaGAP1 mRNA declined in the case of the  $\Delta ras/\Delta ras$  and  $\Delta acpr1/\Delta acpr1$  null mutants and remained unaffected in the N-2-1-6, N-2-1-6 + p33, HLC67 and AS1 strains (Fig. 5e). This implies that Acpr1p/Cph1p-mediated Ras1p signalling regulates CaGAP1 whereas the cAMP-dependent protein kinase A and Efg1p-mediated



**Fig. 5.** Differential expression of *CaGAP1*. (a) A 938 bp *EcoRV* fragment from pCaGAP1 used as probe. (b) Induction of *CaGAP1* by GlcNAc. Total RNA was isolated from strain SC5314 grown in GPK or NPK for 2 h at 30 °C. A Northern blot of the sample was hybridized with the 938 bp *EcoRV* fragment of pCaGAP1. Each lane contains 40  $\mu$ g RNA. (c) Effect of GlcNAc induction in different nitrogen sources. Total RNA was isolated from SC5314 cells in the presence of GlcNAc (2%) in different synthetic media, SN, SEN, SPN, SAN, SUN and SGN, as described in Methods and examined by Northern blot analysis. The same gene probe was used here, and each lane contained 40  $\mu$ g RNA. (d) *CaGAP1* expression levels in media containing alternative nitrogen sources at the 2 h time point were quantified by densitometry of the autoradiogram and fold induction was represented graphically with respect to synthetic complete GlcNAc medium (SN). (e) Effect of GlcNAc induction in different mutants of *C. albicans*. Total RNA was isolated from different strains of *C. albicans*, SC5314, N-2-1-6, N-2-1-6+p33, CAN52, HLC67, AS1 and A-11-1-1-4, in SC medium in the presence of GlcNAc (2%) as described in Methods and examined by Northern blot analysis.

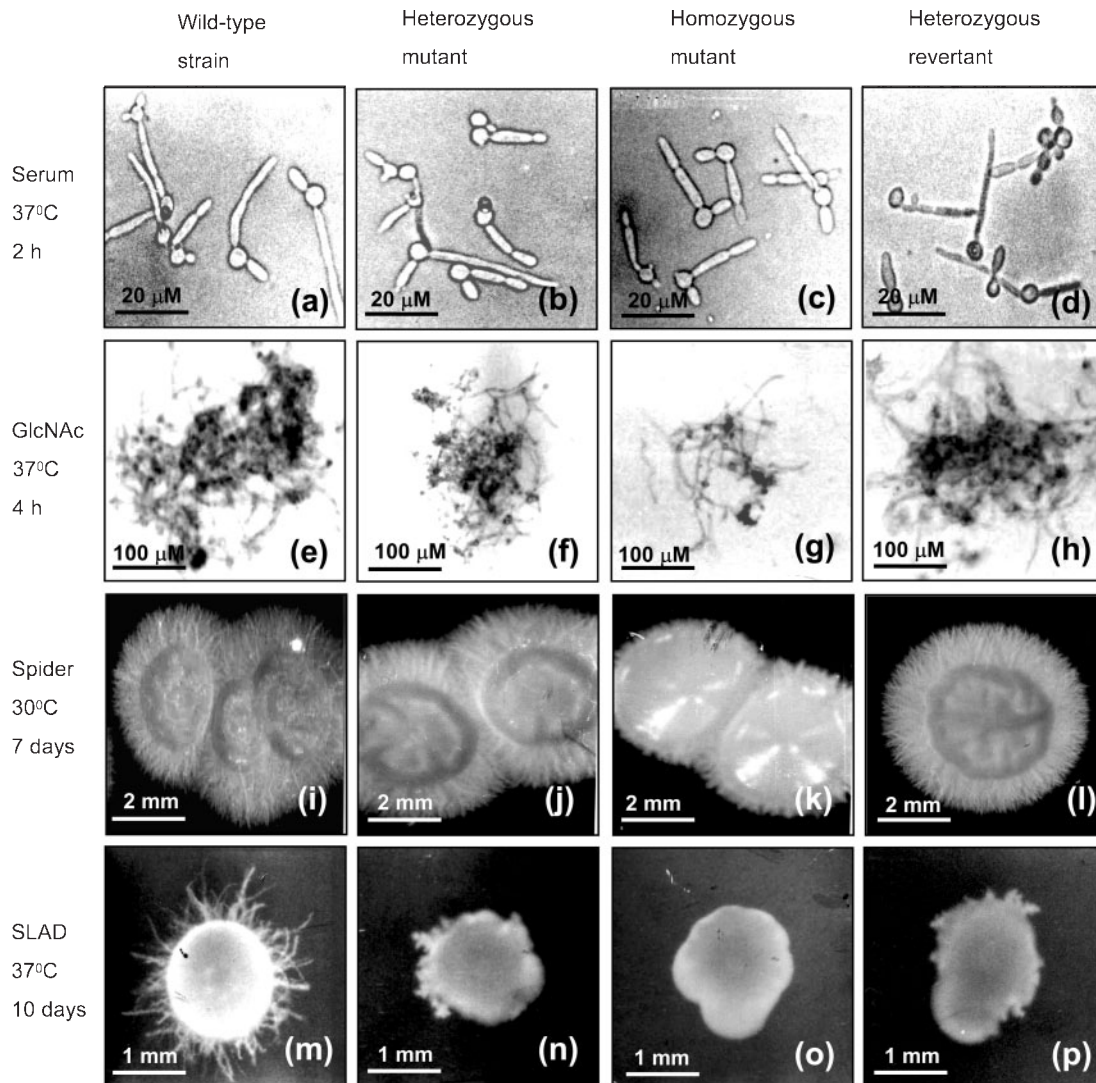
Ras1p signalling pathway is not involved in *CaGAP1* expression. Although *DAC1*, *NAG1* and *HXX1* are induced by GlcNAc, these GlcNAc catabolic pathway genes are not involved in *CaGAP1* expression.

### Physiological effect of disruption of the *CaGAP1* gene

To determine the role of *CaGAP1* in the physiology of *C. albicans*, we disrupted both chromosomal copies of the gene sequentially by the URA-blaster technique (Fonzi & Irwin, 1993). Growth rates of the wild-type (SC5314), heterozygous mutant (GP5), homozygous mutant (GP573) and heterozygous revertant (GP57315) were similar at 30 °C in glucose-containing media (data not shown). In a murine mouse model, no change in virulence was observed with the mutant strains (data not shown). When GlcNAc was used as a carbon source, the growth rate was higher but no

striking difference was found among the wild-type and mutant strains. All the strains used for growth kinetics and morphological studies were Ura<sup>+</sup>. *C. albicans* can shift from a yeast to a hyphal form when it is cultured at 37 °C in the presence of serum and GlcNAc. This transition was not impaired or affected in a *Cagap1/Cagap1* mutant (GP573) in both serum (Fig. 6a, b, c, d) and GlcNAc (Fig. 6e, f, g, h) induction media. However, we observed less hyphal clump formation by GlcNAc in the *Cagap1/Cagap1* mutant in a shake flask (Fig. 6g). No difference was found in a heterozygous mutant and a heterozygous revertant with respect to this behaviour (Fig. 6f, h). We then assessed the filamentous growth from mature colony borders on solid Spider agar in which mannitol, but not glucose, is used as a carbon source at 30 °C. Only the *Cagap1* null mutant (GP573) showed less hyphal formation and altered colony morphology which was different from the wild-type strain and the heterozygous mutant (Fig. 6i, j, k).





**Fig. 6.** Morphology of *CaGAP1* mutant strains under different hypha-inducing conditions. Wild-type SC5314 (*CaGAP1/CaGAP1*), heterozygous mutant GP5 ( $\Delta$ *Cagap1/CaGAP1*), homozygous mutant GP573 ( $\Delta$ *Cagap1/\Delta**Cagap1*) and a heterozygous revertant ( $\Delta$ *Cagap1/\Delta**Cagap1* strain in which *CaGAP1::URA3* has been recombined back at the *CaGAP1* locus,  $\Delta$ *Cagap1/CaGAP1::URA3*) were induced for filamentation under different conditions. *Cagap1/Cagap1* could not block the induction of filaments by serum response (a, b, c, d; bar, 20  $\mu$ m) but shows less hyphal clump formation in GlcNAc-inducing conditions (e, f, g, h; bar, 100  $\mu$ m) and defective filamentation on solid Spider and SLAD media (i, j, k, l; bar, 2 mm). Nitrogen starvation (SLAD medium) (m, n, o, p; bar, 1 mm) also affects filamentation in the case of the heterozygous mutant and revertant strains of *CaGAP1*.

This phenotype was reversed by reconstituting a single functional copy of the gene (Fig. 6l). An interesting feature of our analysis was the finding that both the heterozygous and homozygous mutants had an obvious defect in filamentation and drastic abnormal colony morphology on nitrogen-starvation solid SLAD plates at 37 °C (Fig. 6n, o). Furthermore, the defect in filamentation and colony morphology is not fully suppressed by introduction of a single copy of a functional gene (Fig. 6p). However, *Cagap1/Cagap1* homozygous disruptants were more homogeneous than the heterozygous strain and showed a greater reduction in peripheral hyphal growth,

indicating that gene dosage is important for morphogenesis of *C. albicans* under certain conditions.

## DISCUSSION

We have isolated the general amino acid permease gene *CaGAP1* from *C. albicans*, on the basis of its induction by GlcNAc. This is the first report of the isolation of a functional general amino acid permease gene from *C. albicans*. The results presented here are in agreement with three essential points: first, the activity of a general amino acid permease was regained when a *gap1* mutant

strain of *S. cerevisiae* was transformed with the *CaGAP1* gene in minimal proline media, indicating the functional similarity of CaGap1p with Gap1p; second, transcription of *CaGAP1* is regulated by the external nitrogen source and is dependent on Cph1p-mediated Ras1p signalling; and finally defective filamentation or abnormal colony morphology in homozygous and heterozygous *CaGAP1* disruptants was found under certain conditions.

*CaGAP1* (AF467941) is not only homologous to *GAP1* (CAA82113) of *S. cerevisiae* but also shows similarity to other yeast permease genes such as *HIP1*, *TAT2*, *AGP1* and *GPNI* (Jauniaux & Grenson, 1990). The deduced gene product is highly hydrophobic with 10–12 transmembrane regions. *CaGAP1* was induced by GlcNAc at 2 h growth but was expressed only at a basal level in glucose-containing complete medium. The GlcNAc induction of *CaGAP1* was enhanced in synthetic minimal media supplemented with a single amino acid such as glutamate, proline, glutamine or urea but was inhibited by ammonia. The regulation of *CaGAP1* at the level of transcription is comparable to *GAP1* regulation in yeast, where the transcription factors Gln3p (in the presence of glutamate) and Nil1p (in the presence of urea or proline) are activators (Stanbrough *et al.*, 1995), while Dal80p (Cunningham & Cooper, 1993) and Nil2p (Lodish, 1988; Rowen *et al.*, 1997) are inhibitors. In the presence of ammonium, Ure2p, another transcriptional repressor, sterically hinders Gln3p from activating *GAP1* (Blinder *et al.*, 1996). These factors bind to an upstream regulatory sequence containing a motif surrounding a core GATA sequence (Springael & Andre, 1998). An obvious similarity between the *CaGAP1* promoter and a nitrogen-regulated gene promoter like *GAP1*, *GLN1*, *GDH2*, etc., of *S. cerevisiae* is the presence of a GAATAG sequence (Cunningham & Cooper, 1993). Another feature common to the *CaGAP1* and *GAP1* promoters is the presence of TTGGT or TTGTT, which plays an auxiliary note in activation by Gln3p (Miller & Magasanik, 1991). Five GATA-type transcription factors and one gene homologous to *URE2* have been reported from the *C. albicans* Genome Sequencing Project, Stanford. One can therefore presume that the regulation of *CaGAP1* might be brought about by all of them.

In our induction studies we also saw that *CaGAP1* is GlcNAc-inducible, but in the GlcNAc catabolic pathway mutants  $\Delta dac1\Delta nag1\Delta h x k 1/\Delta dac1\Delta nag1\Delta h x k 1$  (Nag regulon mutated) and  $\Delta dac1\Delta nag1\Delta h x k 1/DAC1NAG1\Delta h x k 1$  (hexokinase mutant), which are incapable of utilizing GlcNAc (Singh *et al.*, 2001), there was no change in induction of *CaGAP1* when GlcNAc was added to the media. This fact implies that catabolism of GlcNAc is not required for expression of *CaGAP1*, but whether GlcNAc directly enhances the expression of *CaGAP1* or whether it binds to some surface receptor which transmits the signals via some other intermediate proteins is still unknown. However, GlcNAc induction of the *CaGAP1* gene is less in *cph1/cph1* and *ras1/ras1* null mutants while no striking

change of expression was found in *efg1/efg1* and *tpk2/tpk2* mutant strains. It was also reported that the N-terminal region of Acprp/Cph1p can recognize and bind PREs *in vitro* like Stel2p of *S. cerevisiae* (Malathi *et al.*, 1994). Interestingly, one PRE sequence, TGAAACA, is also present in the *CaGAP1* promoter. This clearly showed the role of Cph1p-dependent Ras1p signalling in GlcNAc-induced *CaGAP1* expression.

Gap1p of *S. cerevisiae* is not only regulated transcriptionally but its activity also depends on the external nitrogen source. Addition of ammonium ions (Springael & Andre, 1998; Bernard & Andre, 2001) or glutamate (Roberg *et al.*, 1997) inhibits the activity of Gap1p in *S. cerevisiae*. We found in our study that mimosine inhibited the growth of a wild-type strain and transformants ( $\Delta gap1::CaGAP1$ ) of *S. cerevisiae* on minimal proline media but was unable to do so in ammonium- or glutamate-containing media. This indicates that CaGap1p is probably not functional in ammonia- or glutamate-grown cells. Similarly in *C. albicans*, mimosine affected the growth of wild-type strain SC5314 and the revertant strain (GP57315) while a *Cagap1* null mutant (GP573) could resist the drug effect in minimal proline medium. In *Candida* strains, the effect of mimosine persists for a maximum of 10–15 h, which may be because of the higher growth rate of this micro-organism.

Yeast possesses many amino acid permeases with overlapping substrate specificities. The general amino acid permease Gap1p, which can transport most amino acids, can be specifically assayed by uptake of [<sup>14</sup>C]citrulline (Grenson *et al.*, 1970). To demonstrate the import of amino acids by CaGap1p, a citrulline uptake assay was performed in minimal proline medium. General amino acid activity was increased 2.5-fold when the *CaGAP1* gene was expressed in the *gap1* mutant strain ( $\Delta gap1::CaGAP1$ ) of *S. cerevisiae*. On the other hand, the *Cagap1* mutant (GP573) of *C. albicans* showed 50% less citrulline uptake than the wild-type strain (SC5314) and the permease activity was regained when the *CaGAP1* gene was recombined back in the *CaGAP1* locus of the *Cagap1* mutant strain (GP57315). Therefore, we could not exclude the possibility that the transport pattern of the general amino acid permease is the same in both *S. cerevisiae* and *C. albicans*.

We have also shown here that *Cagap1/Cagap1* has defects in filamentation on solid Spider and SLAD medium, forming only a few short hyphae instead of the florid filaments that emanate from the wild-type strain. Despite this defect, *Cagap1/Cagap1* could not block the induction of filaments by serum response, but we found less hyphal clump formation in GlcNAc inducing conditions. Defective morphology and less filamentation of both the heterozygous and homozygous mutants during nitrogen starvation strongly suggest that the GlcNAc-inducible *CaGAP1* is regulated by the external nitrogen source. Thus one interpretation of these data is that GlcNAc-induced hyphal formation is sensitive to the dosage of the *CaGAP1* gene under nitrogen source control. Herein lies

the importance of GlcNAc, which not only acts as an inducer of hyphal formation (Mattia *et al.*, 1982; Simonitti *et al.*, 1974) but also regulates the expression of a number of genes within the cell. Through the induction of *CaGAP1*, GlcNAc might indirectly alter the nutritional status of the cell, by causing an increased uptake of amino acids. Again, depending on the source of nitrogen in the extracellular medium, *CaGAP1* is induced or repressed. In a poor nitrogen source like minimal proline medium or under nitrogen starvation conditions, *CaGAP1* is induced by GlcNAc through the Cph1p-mediated Ras1p signalling pathway, which leads to a morphological change. This interplay between GlcNAc and different nitrogen sources probably brings about a co-ordinated regulation of *CaGAP1* expression and morphogenesis.

## ACKNOWLEDGEMENTS

The authors wish to thank M. C. Brandriss, G. R. Fink, J. F. Ernst, W. A. Fonzi and Q. Feng for providing strains and M. Sengupta for stimulating discussions. We thank K. Natarajan for valuable suggestions during the course of this work. We are grateful to A. Chattopadhyay and A. Sengupta for help with microscopy. This work was supported by grants from the Department of Biotechnology and Council of Scientific and Industrial Research.

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