Chapter 3

CLONING, COMPLEMENTATION, EXPRESSION AND MUTATION STUDIES OF CaGAPI GENE IN C. ALBICANS
3. 0. INTRODUCTION

The ability to use a variety of nitrogen containing compounds as the sole source of all cellular nitrogen is a predominant feature in Yeast. This ability requires permeases for transport of nitrogenous compounds and enzymes for the generation of ammonia by their metabolic process. In response to changes in the environment, there occurs an increase in the activity of the permeases responsible for uptake of amino acids for use as nitrogen source. This is also true for the opportunistic yeast Candida albicans which is the leading etiological 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 signaling 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 pathway (Feng et al., 1999). In this pathway, two catalytic subunits or isoforms of protein kinase A (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 efg1lefg1 defect in hyphal development, whereas overexpression of EFG1 can suppress the filamentation defect in tpk2tpk2, which implies 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/Acp1p, a homolog of Ste12p (Liu et al., 1994; Singh et al., 1994; Singh et al., 1997), and MAP kinase cascade that includes Cst20p (p21 activated kinase; PAK) (Leberer et al., 1996; Leberer et al., 1997), Hst7p (MAP kinase kinase; MEK) (Leberer et al., 1996) and 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 yeast to hyphal form (Singh et al., 2001). Filamentation regulated by Nag regulon is (HXK1/DAC1/NAG1) independent
Cloning, complementation, expression and mutation studies of CaGA\(^1\) gene in Candida albicans

of Tpk2p and Cphlp/Acprlp regulated MAP kinase pathway but is dependent on the morphological regulator Efglp (Ghosh et al., 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 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, CaGA\(^1\), which is homologous to GAP1, 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 (Sophianopoulou and Diallionas, 1995). In presence of ammonia or glutamine, the amino acid uptake is less whereas in media containing 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 5 proteins (Ure2p, Dal80p, Gln3p, Nil1p and Nil2p) function coordinately 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) (see details in next section 3.1 and Fig 3.3)

This chapter deals with Complementation studies by expressing CaGA\(^1\) in gap1 mutant of S. cerevisiae which showed the functional similarity of Cagaplp with the general amino acid permease (Gap1p) of S. cerevisiae. We observed a differential expression of CaGA\(^1\) in various nitrogen sources as well as in mutants defective for morphogenesis and virulence. We also report here some conditions where filamentation and morphogenesis were altered in heterozygous and homozygous disruptants of CaGA\(^1\).

3.1. Nitrogen regulation and general amino acid permease activity in S. cerevisiae

3.1.1. Nitrogen Regulation (Nitrogen Catabolic Repression)

Nitrogen catabolic repression is the mechanism designed to prevent or reduce the unnecessary divergence in the synthetic capacity of the cells to the formation of enzymes and permeases for the utilization of compounds that are non-preferred sources of glutamate and glutamine when a preferred nitrogen source is available (Magasanik and Kaiser, 2002). Regulations of the general amino acid control are achieved by certain amino acid permeases by SSY1 pathway for sensing amino acids in the medium.
3.1.2. The core pathway for nitrogen assimilation

When cells have an abundant source of ammonia, either by conversion of a non-preferred nitrogen source to ammonia or by growth on ammonia itself, the NADP⁺-linked glutamate dehydrogenase, the product of GDH1, is responsible for the synthesis of glutamate by combining ammonia with the citric acid cycle intermediate α-ketoglutarate (Grenson et al., 1974)

\[
\alpha\text{-ketoglutarate} + NH_4^+ + NADPH + H^+ \leftrightarrow \text{glutamate} + NADP^+
\]

Glutamate can then combine with ammonia in a reaction catalyzed by glutamine synthetase, the product of GLN1 (Mitchel and Magasanik, 1983; Mitchel, 1985) (Fig. 3. 1).

\[
\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{glutamine} + \text{ATP} + P
\]

The entry of ammonia into the cell is facilitated by three permeases, the products of MEP1, MEP2, and MEP3. The high affinity permeases MEP1 and MEP2 also appear to play a role in the intracellular retention of ammonia generated by the degradation of arginine and urea (Magasanik and Kaiser, 2002) (Fig. 3. 1).

When glutamate is the sole source of nitrogen, the NAD⁺-linked glutamate dehydrogenase, the product of GDH2, is responsible for the release of ammonia required for the synthesis of glutamine from glutamate (Miller and Magasanik, 1991)

\[
\text{Glutamate} + \text{NAD}^+ \leftrightarrow \text{NAD}^+ + \text{NADH} + H^+ + \alpha\text{-ketoglutarate}
\]

Glutamate synthetase is the only means by which glutamine can be synthesized and cells lacking this enzyme require glutamine for growth. On the other hand, the loss of GDH1 only reduces the rate of growth on ammonia by one half (Wiame et al., 1985). The ability of gdh1 mutants to grow on ammonia is due to the existence of the enzyme glutamate synthase, the product of GLT1, which catalyzes the following reaction (Miller and Magasanik, 1990; Cogoni et al., 1995) (Fig. 3. 1).

\[
\text{Glutamine} + \alpha\text{-ketoglutarate} + \text{NADH} + H^+ \rightarrow 2 \text{glutamate} + \text{NAD}^+
\]

Reactions (2) and (4) together catalyze the net synthesis of glutamate from α-ketoglutarate and ammonia. Reaction (3) is not required in cells growing on ammonia, but is essential for the synthesis of glutamine in cells using glutamate, or nitrogen compounds which are precursors of glutamate such as proline or γ-aminobutyrate as a source of nitrogen (Miller and Magasanik, 1990). Reactions (1) to (4) catalyze the final steps in the conversion of non-preferred sources of nitrogen to glutamate and glutamine, which in turn serve as the source of all cellular nitrogen. Consequently, an increase in the level of these enzymes is required to compensate for the low intracellular concentrations of their substrates in cells growing on non-preferred sources of nitrogen (Fig. 3. 1).
Fig. 3. 1. Central pathways for nitrogen metabolism. The nitrogenous compounds in the cell are synthesized from either glutamate or glutamine. The major pathway for glutamate synthesis is through combination of ammonia with $\alpha$-ketoglutarate, which is synthesized from acetyl CoA and oxaloacetate through the early steps of the citric acid cycle. Glutamine is synthesized by the combination of ammonia with glutamate. The pathways for utilization of a variety of nitrogen sources, including urea, proline and arginine, are shown. The *Saccharomyces cerevisiae* gene for each of the enzymatic steps is designated in italics (Magasanik and Kaiser, 2002).
In some cases, the enzymes responsible for the utilization of a non-preferred source of nitrogen are induced by the presence of the particular nitrogen source in the growth medium. For example, expression of the genes coding for the enzymes needed for the utilization of arginine and proline as sources of nitrogen are induced by the presence in the growth medium of arginine and proline, respectively. Strong activation by arginine depends on the activators Arg80p and Arg81p, whereas strong activation by proline depends on Put3p.

Preferred nitrogen sources
- Ammonia, glutamine, asparagine, mixture of amino acids and peptides in commercial Bactopeptone.

Non preferred sources of nitrogen
- Proline, Ornithine, γ-aminobutyrate, allantoin and urea.

Note: Nitrogen sources that lead to derepression of alternative pathway are considered to be non-preferred.

3.1.3. The elements of nitrogen regulation

H. Holzer and his coworkers made the initial observations leading to the concept of nitrogen regulation in S. cerevisiae. They showed that the intracellular levels of the NAD⁺-linked glutamate dehydrogenase and of glutamine synthetase were much lower in cells grown with ammonia, glutamine, or asparagine than in those grown with glutamate or aspartate as sources of nitrogen (Magasanik and Kaiser, 2002). Subsequently, Grenson, Hou and Crabbeel reported the discovery of a general amino acid permease (GAP) present in cells grown with proline, but not in those grown with ammonia as a source of nitrogen (Grenson et al., 1970).

A common genetic basis for nitrogen regulation was suggested by the discovery in 1972 of a recessive mutation known as ure2. In addition, the ure2 mutation prevented the inactivation of glutamine synthetase, when glutamine was added to wild type cells grown with glutamate as a source of nitrogen (Legrain, 1982; Coschigano and Magasanik, 1991.). The GLN3 gene was identified by a mutation that resulted in a phenotype opposite to that of ure2. By starting with a gln1 mutant that could grow slowly in the absence of glutamine, a gln3 gln1 double mutant was selected by its total inability to grow in the absence of glutamine (Mitchell and Magasanik, 1984). When combined with a functional structural gene for glutamine synthetase, the gln3 mutant grew in the absence of glutamine, but was unable to produce glutamine synthetase at a high level when grown with glutamate as a source of nitrogen. The gln3 mutation was pleiotropic: the gln3 mutant failed to produce NAD⁺-linked glutamate dehydrogenase and four other proteins, identified by two-
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

dimensional gel analysis, at a high level when grown with glutamate as a source of nitrogen (Mitchell and Magasanik, 1984).

Evaluation of the phenotype of gln3 ure2 double mutants revealed an inability to increase the level of glutamine synthetase and of the NAD⁺-linked glutamate dehydrogenase in response to growth with glutamate as a source of nitrogen. Taken together, all of these data suggested that the product of GLN3 activated the formation of these enzymes and that the product of the URE2 gene blocked this activation in response to glutamine (Coschigano and Magasanik, 1991). The role of Gln3p is the activation of the initiation of transcription at the promoters of nitrogen-regulated genes. The role of Gln3p as the agent responsible for the activation of the expression of nitrogen-regulated genes was confirmed by the finding that the transcription of the genes for allantoin utilization (DUR1, DUR2, DAL5, and DAL7) did not occur in cells grown with asparagine as a source of nitrogen, and required a functional GLN3 gene in cells grown with proline as a source of nitrogen (Cooper et al., 1990).

The analysis of the DNA sequence located upstream from the DAL5 gene suggested that the site (UAS₇) responsible for nitrogen regulation of the transcription of this gene had the sequence GATAAG (Rai et al., 1989). Immunoprecipitation experiments with Gln3p polyclonal antibody indicated that Gln3p binds to a site located upstream of GLN1 (Minehart and Magasanik, 1991), and immunoprecipitation experiments also showed that Gln3p could bind to the regulatory factor Ure2p (Blinder et al., 1996). Another protein with a zinc finger closely related to that of Gln3p was discovered by its inhibitory effect on the expression of some, but not all, genes responsive to nitrogen regulation: the loss of the product of the DAL80 (UGA43) gene was found to result in greatly enhanced expression of the genes coding for some of the permeases and enzymes responsible for the degradation of urea and allantoin (DAL) and for those responsible for the degradation of γ-amino butyrate (UGA) in cells grown with proline, but not in those grown with asparagine or ammonia as a source of nitrogen. The expression of the DAL80 gene itself was found to be subject to nitrogen regulation (Chisholm and Cooper, 1982; Vissers et al., 1990; Cunningham and Cooper, 1991).

The study of the expression of the GAP1 gene, coding for the general amino acid permease, revealed the existence of a gene responsible for the activation of GAP1 expression in cells grown with urea or ammonia, but not in cells grown with glutamate as a source of nitrogen. This gene, NIL1 (GAT1), has a zinc finger highly homologous to that of Gln3p and Dal80p. In contrast to
Fig. 3.2. Regulatory circuit of transcription of GAP1 in S. cerevisiae involving the inducible and inhibitory action of GATA type transcription factors. The GATA-type transcription factors Gln3p (in presence of glutamate) and Nil1p (in presence of urea or proline) are activators of GAP1 expression, while Dal80p and Nil2p are inhibitors. In the presence of ammonium, Ure2p, another transcriptional repressor sterically hinders Gln3p from activating GAP1 (Lodish, 1988; Rowen et al., 1997; Cunningham and Cooper, 1993; Stanbrough et al., 1995 Blinder et al., 1996).
GLN3, which is expressed constitutively regardless of the nitrogen source, the NIL1 gene is subject to nitrogen regulation and requires Gln3p or Nil1p for its expression (Stanbrough et al., 1995). The transcriptional regulation of nitrogen control genes including GAP1 is given in Fig. 3. 2.

3. 1. 4. Transcription factors and their targets

The four transcription factors share a characteristic zinc finger region which apparently permits them to bind to a region of DNA, UASN, located several hundred base pairs upstream from the genes subject to nitrogen regulation and to activate (in the case of Gln3p and Nil1p) or to block (in the case of Dal80p and Nil2p) the initiation of transcription. The highest homology, 80% based on the identity of amino acid residues, is found in the region of 52 amino acid residues overlapping the zinc fingers of Nil1p, Nil2p and Dal80p; the corresponding region of Gln3p is 65% homologous to that of the other three GATA factors (Stanbrough et al., 1995). The only other zinc finger region with some homology to that of the GATA factors is found in Ash1p, a protein that plays a role in the repression of the HO endonuclease gene in daughter cells after cell division (Bobola et al., 1996). The zinc finger region of Ash1p differs from that of the GATA factors by the presence of three additional amino acid residues in the region separating the two zinc fingers and is only 35% homologous to the GATA factors. It seems therefore that Gln3p, Nil1p, Nil2p, and Dal80p are the only transcription factors able to recognize the GATA sequences of the nitrogen-regulated promoters.

The two positive transcription factors, Gln3p and Nil1p, differ from the negative transcription factors, Nil2p and Dal80p, by having a homologous region, located approximately 200 amino acid residues from the amino-terminus that is exceptionally rich in asparagine residues (Stanbrough et al., 1995). On the other hand, Nil2p and Dal80p share a carboxyl terminal domain containing a leucine zipper, which is not found in Gln3p or Nil1p, and may be responsible for the ability of these proteins to dimerize (Cunningham et al., 2000; Coffman et al., 1997).

The evidence for specific binding to its target is strongest for Gln3p. This protein, overproduced in cells of S. cerevisiae and purified as a tetramer of 500 kDa, was shown to retard the gel migration of DNA preparations containing the 5'-GATAAGATAAG-3' and 5'-GATTAGATTAG-3' sequences located upstream of the GLN1 and GDH2 genes, respectively. The sequence 5'-GATAA-3' found upstream from most nitrogen-regulated genes, usually as 5'-GATAAG-3', is a strong binding site, the sequence 5'-GATTAGATTAG-3' located upstream from GDH2 is a weaker
binding site, and the sequences 5'-GATGAT-3' and 5'-GATAGT-3' fail to serve as binding sites. Although many of the DNA preparations used in these experiments contained more than one GATAAA sequence, the presence of a single GATAAG, GATAAGATAAG or GATTAGATTAG sequence was adequate for binding the degradation product(s) of Gln3p (Cunningham et al., 2000).

3.1.5. Nitrogen-regulated intracellular sorting of amino acid permeases

Saccharomyces cerevisiae encodes 19 amino acid permeases identifiable as members of a diverse family of transporters for amino acids, polyamines and choline (APC) found in bacteria, fungi and mammalian cells (Nelissen et al., 1997). The amino acid permeases are integral membrane proteins with 12 predicted transmembrane domains which are delivered by the secretory pathway to the plasma membrane where they function to take up amino acids for protein synthesis and for use as sources of nitrogen (Andre, 1995). These permeases can be divided into two classes according to their regulation and function (Sophianopoulou and Diallinas, 1995). The nitrogen-regulated permeases include Gap1p, which transports all naturally occurring amino acids (Jauniaux and Grenson, 1990), and Put4p, which transports only proline (Lasko and Brandriss, 1981; Vandenbol et al., 1990). Both of these permeases are coordinately derepressed during growth on poor nitrogen sources, implying that their function is to provide the cell with amino acids to be used as a source of nitrogen (Wiame et al., 1985). Members of the other class of permeases are expressed even when cells are grown on a preferred source of nitrogen. Most of these permeases are specific for particular amino acids, or chemically related sets of amino acids, such as the histidine permease, Hip1p (Tanaka and Fink, 1985), the basic amino acid permease, Can1p (Hoffmann, 1985), and Tat2p, a tryptophan permease (Schmidt et al., 1998). This permease class is thought to transport amino acids that are available in the growth medium for use in protein synthesis. List of permeases is given in Table 3.1.

- Plasma-membrane general amino acid permease transports all naturally-occurring L-amino acids (Km in the range of 3 micromolar to 1 millimolar), GABA, ornithine, citrulline (Km of 80 micromolar), some D-amino acids, and other toxic analogs.
- This permease is most active in cells grown on proline or urea as sole nitrogen source. On such media, transcription of the GAP1 gene involves the general nitrogen positive regulatory factors Gln3p and Nil1p/Gat1p.
- On preferred nitrogen sources like ammonia, expression of the GAP1 gene is subject to nitrogen catabolite repression. This repression involves the Ure2p/GdhCRp and Gzf3p/Nil2p/Deh1p negative factors.

119
<table>
<thead>
<tr>
<th>High affinity histidine permease (also implicated in Mn$^{2+}$ efflux; Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ uptake)</th>
<th>Yeast, fungi</th>
<th>Hip1 of <em>Saccharomyces cerevisiae</em> (spP06775)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General amino acid permease</strong> (all L-amino acids and some D-amino acids)</td>
<td>Yeast</td>
<td>Gap1 of <em>Saccharomyces cerevisiae</em> (spP19145)</td>
</tr>
<tr>
<td>Proline permease</td>
<td>Yeast</td>
<td>Put4 of <em>Saccharomyces cerevisiae</em> (spP15380)</td>
</tr>
<tr>
<td>Arginine permease</td>
<td>Yeast</td>
<td>Can1 of <em>Saccharomyces cerevisiae</em> (spP04817)</td>
</tr>
<tr>
<td>High affinity glutamine permease</td>
<td>Yeast</td>
<td>Gnp1 of <em>Saccharomyces cerevisiae</em> (spP48813)</td>
</tr>
<tr>
<td>Leu/Val/Ile amino acid permease</td>
<td>Yeast</td>
<td>Bap2 of <em>Saccharomyces cerevisiae</em> (spP38084)</td>
</tr>
<tr>
<td>Asn/Gln permease</td>
<td>Yeast</td>
<td>Agp1 of <em>Saccharomyces cerevisiae</em> (spP25376)</td>
</tr>
<tr>
<td>Tryptophan permease</td>
<td>Yeast</td>
<td>Tat2 of <em>Saccharomyces cerevisiae</em> (spP38967)</td>
</tr>
<tr>
<td>Val/Tyr/Trp permease</td>
<td>Yeast</td>
<td>Val1 (Tat1) of <em>Saccharomyces cerevisiae</em> (spP38085)</td>
</tr>
<tr>
<td>Lysine permease</td>
<td>Yeast</td>
<td>Lyp1 of <em>Saccharomyces cerevisiae</em> (spP32487)</td>
</tr>
<tr>
<td>Basic amino acid permease</td>
<td>Yeast</td>
<td>Alp1 of <em>Saccharomyces cerevisiae</em> (spP38971)</td>
</tr>
<tr>
<td>Leucine sensor/transcription factor</td>
<td>Yeast</td>
<td>Ssy1 of <em>Saccharomyces cerevisiae</em> (spQ03770)</td>
</tr>
<tr>
<td>Dicarboxylic amino acid permease</td>
<td>Yeast</td>
<td>Dip5 of <em>Saccharomyces cerevisiae</em> (spP53388)</td>
</tr>
<tr>
<td>General amino acid permease with broad specificity, SAM3</td>
<td>Yeast</td>
<td>Agp3 of <em>Saccharomyces cerevisiae</em> (spP43548)</td>
</tr>
<tr>
<td>S-adenosylmethionine uptake permease, SAM3</td>
<td>Yeast</td>
<td>SAM3 (YPL274w) of <em>Saccharomyces cerevisiae</em> (587aas; pirS65307)</td>
</tr>
<tr>
<td>S-methylmethionine uptake permease, Mmp1</td>
<td>Yeast</td>
<td>Mmp1 (YLL061w) of <em>Saccharomyces cerevisiae</em> (583aas; pirS0959)</td>
</tr>
</tbody>
</table>

Amino-acid permeases most likely act as H$^{+}$ symporters.
Ammonia triggers loss of activity and degradation of the Gap1 permease. This regulation involves the essential Npl1p/Rsp5p ubiquitin-ligase. The latter enzyme is also involved in ubiquitination and degradation of permeases Fur4p and Mal61p.

Mutations in the cytosolic C-terminal region of Gap1 protects the permease against ammonia-induced inactivation and degradation. One mutation affects a di-leucine; another a DAKSS-like sequence; deletion of the last 11 aa also abolishes this regulation.

The general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in S. cerevisiae.

3.1.6. Post-transcriptional regulation of general amino acid permease, Gap1p

*GAP1* regulation followed the Gap1p activity after transfer of a yeast culture growing on proline as a nitrogen source to a medium containing ammonia. The Gap1p activity as measured by cellular uptake of radio-labeled citrulline is high in cells grown on proline medium. Some of the decline in Gap1p activity could be attributed to a cessation in *GAP1* transcription brought about by the inactivation of transcriptional activators Gln3p and Nlr1p by the presence of ammonia. However, a *ure2* mutant which transcribes *GAP1* constitutively also exhibited full inactivation by ammonia. When *GAP1* is expressed from a heterologous constitutive promoter, which does not respond to the nitrogen source, the amount of Gap1p permease activity nevertheless registers the full response to growth on glutamate or glutamine as a nitrogen source, showing that much of the observed nitrogen regulation of Gap1p transport activity can be attributed to post-transcriptional regulation (Chen and Kaiser, 2002).

Experiments following the intracellular location of Gap1 protein showed that the mechanism of post-transcriptional regulation of Gap1p was a consequence of regulated protein sorting in the late secretory pathway. Fractionation of cell membranes carried out in parallel with the activity measurements demonstrated that in cells grown on glutamate, Gap1p is located in ER and Golgi compartments but not in the plasma membrane. Under these conditions Gap1p is transported to the vacuole without ever being delivered to the plasma membrane. When cells grown on glutamate are transferred to urea medium a dramatic increase in Gap1p activity is accompanied by a redistribution of Gap1p protein to the plasma membrane (Roberg et al., 1997). Redistribution of Gap1p to the plasma membrane depends on the function of secretion genes such as *SEC6*, which is required for fusion of post-Golgi secretory vesicles with the plasma membrane. Thus, the activity of Gap1p permease appears to be regulated largely by the amount of Gap1p that is located in the plasma membrane and the key process that controls delivery of Gap1p to the plasma membrane.
Fig. 3. Intracellular trafficking pathways of Gap1p. Conditions of nitrogen limitation can trigger dephosphorylation of cytoplasmic Gln3p and release from Ure2p. With the aid of the nuclear import factors, Gln3p can then enter the nucleus where it can activate transcription of nitrogen-regulated genes. The nitrogen-regulated permease Gap1p is transported through the secretory pathway to the Golgi complex. In the Golgi, ubiquitination of Gap1p by the E3 ubiquitin ligase complex consisting of Rsp5p, Bul1p and Bul2p causes Gap1p to be diverted to the prevacuolar compartment (PVC) and the vacuole. Conditions of nitrogen limitation allow Gap1p to be recycled to the Golgi and to the plasma membrane. Recycling may involve the action of the Npr1p kinase and the membrane protein Lst4p.

Table 3.2. Genes that control Gap1p permease sorting

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant Phenotype</th>
<th>Essential Features</th>
<th>Reference</th>
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<tbody>
<tr>
<td>RSP5/NPI1</td>
<td>Gap1p in PM</td>
<td>Yes</td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td>DOA4/NPI2</td>
<td>Gap1p in PM</td>
<td>No</td>
<td>Deubiquitinating enzyme</td>
</tr>
<tr>
<td>BUL1, BUL2</td>
<td>Gap1p in PM</td>
<td>No</td>
<td>Binds to Rsp5p</td>
</tr>
<tr>
<td>NPR1</td>
<td>Gap1p in vacuole</td>
<td>No</td>
<td>Ser/Thr kinase</td>
</tr>
<tr>
<td>SEC13</td>
<td>Gap1p in vacuole</td>
<td>Yes</td>
<td>COPII subunit</td>
</tr>
<tr>
<td>LST4</td>
<td>Gap1p in vacuole</td>
<td>No</td>
<td>Integral membrane protein</td>
</tr>
<tr>
<td>LST7</td>
<td>Gap1p in vacuole</td>
<td>No</td>
<td>Hydrophilic protein</td>
</tr>
<tr>
<td>LST8</td>
<td>Gap1p in vacuole</td>
<td>Yes</td>
<td>Peripheral membrane protein</td>
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Cloning, complementation, expression and mutation studies of CaGAP1 gene in *Candida albicans*

during the nitrogen-dependent sorting of Gaplp in the intracellular Golgi and prevacuolar (PVC) compartments. Strategy of posttranscriptional regulation and the list of the genes involved in sorting of Gaplp is given in Fig. 3. and Table 3.2.

Gaplp activity after transfer to ammonia is accompanied by degradation of Gaplp protein in the vacuole suggested that the endocytosis of Gaplp is largely responsible for ammonia inactivation (Hein and André, 1997). It was further proposed that ammonia inactivation is the result of a greatly increased rate of Gaplp endocytosis in response to ammonia (Springael and André, 1998). NH₄⁺-induced down-regulation of the *S. cerevisiae* Gaplp permeases involves its ubiquitination with lysine-63-linked chains.

Degradation of many eukaryotic proteins requires their prior modification by conjugation with ubiquitin (Ub). Ub molecules are transferred to lysine residues of target proteins via an E1-E2-E3 enzyme thioester cascade (Ub-activating enzymes/Ub-conjugating enzymes/Ub-protein ligase) (Ciechanover, 1994). Ubiquitination is known mainly as a signal targeting substrate proteins for recognition and degradation by a multisubunit protease, the 26S proteasome (Pickart, 1997).

In this case, we could hypothesize that the quality of the nitrogen source controls both an intracellular sorting event that governs the rate of delivery of Gaplp to the plasma membrane and an endocytic sorting process which determines the rate at which Gaplp is removed from the plasma membrane. An alternative possibility, that is consistent with all of the available data on the regulation of Gaplp sorting, is that Gaplp is endocytosed continuously under all conditions and that only the intracellular sorting which determines the rate of delivery to the plasma membrane is regulated by nitrogen. According to this idea, ammonia inactivation would be the consequence of constitutive endocytosis causing Gaplp to be removed from the plasma membrane after a cessation of new Gaplp delivery to the plasma membrane caused by the effect of ammonia on Golgi sorting of Gaplp. Resolution of the question of whether or not Gaplp endocytosis is regulated by nitrogen must await the development of specific assays that will allow measurement of the rate of Gaplp endocytosis to be measured independently of the rate of delivery to the plasma membrane.

3. 1. 7. General amino acid permease acts as an amino acid sensor

Eukaryotic cells have sensing systems for a variety of extracellular stimuli, including chemical messengers and physical factors. Also, nutrients exert regulatory effects on eukaryotic cells that do not appear to be mediated by their function in energy or substrate provision, but rather by specific
nutrient-sensing mechanisms. Knowledge of these mechanisms is still very limited. Hexokinase in *S. cerevisiae* (Herrero et al., 1998; Hohmann et al., 1999) and *Arabidopsis thaliana* (Sheen et al., 1999) have been recently proposed as glucose sensor. The non-transporting amino acid carrier homologue Ssy1 (Didion et al., 1998; Iraqui et al., 1999; Klasson et al., 1999) and the Mep2 ammonium carrier (Lorenz and Heitman, 1998) have been proposed to play a role as nitrogen sensors in *S. cerevisiae*. Very recently Donaton et al. showed that the general amino acid permease Gap1p in yeast not only functions as an amino acid transporter but also plays a role as an amino acid sensor for rapid activation of the PKA targets upon addition of amino acids to cells starved for nitrogen on a glucose containing medium. Transport of amino acids by the Gap1 permease apparently triggers the initial activation of the pathway upon refeeding of amino acids to nitrogen-starved cells. Specific truncations of the Gap1 C-terminus cause permanent activation of the signaling pathway independent of Gap1 transport activity, and this result in a high-PKA phenotype *in vivo*. This observation indicates that eukaryotic cells not only use specific receptor proteins for nutrient regulation but also use genuine nutrient transporters as receptors for activation of major signaling pathways (Donaton et al., 2003).

3.2. MATERIALS AND METHODS

3.2.1. Strains and Plasmids used

The strains and plasmids used in this study have been listed in Table 3.3.

3.2.2. Media and Solutions

The common media and solutions used have been listed in section 2.6.2. Minimal-Proline medium (MIN-Proline/SPD) contained 0.67 % of Yeast nitrogen base without (NH₄)₂SO₄ and without amino acids, 2 % Dextrose and 1 g proline l⁻¹ (added after autoclaving). This medium was used to select and score the mutation, which conferred resistance to the amino acid analog, mimosine (75μg ml⁻¹). Min-Glutamate (SED), Min-Ammonium (SAD) media contained 1 g glutamate l⁻¹ or 2 g ammonia l⁻¹. All solid media contained 2 g agar l⁻¹. GPK (0.5% Dextrose, 0.5% Peptone and 0.3% KH₂PO₄) and NPK (0.5% GlcNAc, 0.5% Peptone and 0.3% KH₂PO₄) were used for GlcNAc induction studies in *C. albicans*. Nonstandard synthetic media used for analyzing inducing effects of alternate nitrogen sources were glutamate (SEN), proline (SPN), ammonia (SAN), urea (SUN) and glutamine (SGN) containing 0.67 % yeast nitrogen base and 2% GlcNAc with the respective nitrogen source. GlcNAc and the other nitrogen sources glutamate (1 g l⁻¹),
## Table 3. Strains and Plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype/description</th>
<th>source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F′/erm A1 hsd R17 (rK mcrA) glnV44 thi-4 recA1 gyrA (Nal) relA1 Δ(lacIYS4-argF) U169 deoR (φ80d lacΔlacZ)M15</td>
<td></td>
</tr>
<tr>
<td><strong>S. cerevisiae strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS138</td>
<td>MATa ura3-52 leu2-3 GAP1</td>
<td>M.C. Brandriss, NJMS</td>
</tr>
<tr>
<td>MS143</td>
<td>MATa ura3-52 leu2-3 Δgap1::LEU2</td>
<td>M.C. Brandriss, NJMS</td>
</tr>
<tr>
<td>MSPF31</td>
<td>MATa ura3-52 leu2-3 Δgap1::LEU2 [CAGAPl]</td>
<td>This work</td>
</tr>
<tr>
<td><strong>C. albicans strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC5314 (Wild type)</td>
<td>URA3/URA3</td>
<td>W.A. Fonzi</td>
</tr>
<tr>
<td>CAF-3-1(Wild type)</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td></td>
</tr>
<tr>
<td>GP-5</td>
<td>As CAF3-1, but CAGAPl/Δcagap1::hisG-URA3-hisG</td>
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</tr>
<tr>
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<td>As CAF3-1, but CAGAPl/Δcagap1::hisG</td>
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</tr>
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<td>GP-573</td>
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<td>This work</td>
</tr>
<tr>
<td>GP-5731</td>
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<td>This work</td>
</tr>
<tr>
<td>GP-57315</td>
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<td>This work</td>
</tr>
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<td>N-2-1-6</td>
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<td>Laboratory strain</td>
</tr>
<tr>
<td>N-2-1-6-1+p33</td>
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<td>Laboratory strain</td>
</tr>
<tr>
<td>CAN52</td>
<td>Δras1::hisG/Δras1::hph Δura3::imm434/Δura3::imm434</td>
<td>Feng et al.(1999)</td>
</tr>
<tr>
<td>HLC67</td>
<td>Δefgl::hisG/Δefgl::hisG Δura3::imm434/Δura3::imm434</td>
<td>J.F.Ernst(2001)</td>
</tr>
<tr>
<td>AS1</td>
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<td>J.F.Ernst(2001)</td>
</tr>
<tr>
<td>A-11-1-1-4 (cph1')</td>
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<td>Laboratory strain</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pGPORF</td>
<td>CAGAPl ORF cloned in pGEM-T Easy vector</td>
<td>This work</td>
</tr>
<tr>
<td>pFL61</td>
<td>URA marked ScARS vector plasmid</td>
<td>ATCC</td>
</tr>
<tr>
<td>pFLGP31</td>
<td>CAGAPl ORF sub cloned into pFL61 under PGK promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pCaGAP1</td>
<td>3.5kb CAGAPl fragment cloned in pGEM-T Easy vector</td>
<td>This work</td>
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<td>pGP1</td>
<td>carrying Δcagap1::hisG-URA3-hisG disruption fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pGP2</td>
<td>carrying CAGAPl::URA3 reconstruction fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pCUB6</td>
<td>carrying hisG-URA3-hisG disruption cassette</td>
<td>W.A. Fonzi</td>
</tr>
<tr>
<td>pUC19-CUB</td>
<td>hisG-URA3-hisG disruption cassette cloned in pUC19</td>
<td>This work</td>
</tr>
</tbody>
</table>
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

proline (1 g l⁻¹), ammonia (2 g l⁻¹), urea (2 g l⁻¹), and histidine (1 g l⁻¹) were filter sterilized and added after autoclaving. Induction effect of single amino acid or urea was shown here in respect of synthetic complete (SN) GlcNAc medium.

3.2.3. Growth and Maintenance of strains

Candida albicans strain SC5314 was routinely cultured in YPD or SD media (section 2.6.2) at 30°C at 200rpm shaking. For growing the ura3 auxotrophs, such as CAF3-1 and other ura cured transformants of C. albicans, SD medium was supplemented with Uridine at the concentration of 25μg/ml. E. coli strains were cultured in Luria-Bertani broth or LB agar plates containing 50μg/ml and 75μg/ml ampicillin respectively.

3.2.4. Storage of C. albicans and E. coli strains

Bacterial and C. albicans strains were stored as glycerol stocks at minus 80°C. To the overnight grown cultures, sterile glycerol was added to the final concentration of 15%.

3.2.5. Isolation of CaGAP1

CaGAP1 gene was isolated by differential screening (see details in section 2.2.1) of a C. albicans genomic library in Yep13 with cDNA probes synthesized from poly (A⁺) RNA of glucose grown (uninduced) and GlcNAc grown (induced) cells (Okayama et al., 1987). The clone was subsequently sequenced. The sequence data was matched with the C. albicans Genome Sequencing Project, Stanford, followed by ORF analysis through ORF Finder, NCBI. Nucleotide sequence of CaGAP1 is given in Fig. 3.4.

3.2.6. Cloning of the CaGAP1 gene

The CaGAP1 coding region was PCR-amplified from genomic DNA (Isolation process was described in section 2.6.8) of C. albicans SC5314 using the oligonucleotide 5' TGATCCTTTAATCTTGAGAAGG 3'(GP1) and 5'TGTTCAACCTGGTCAAAGTCC 3'(GP2) as primers. The 2.2 kb PCR fragment was cloned into pGEMT-Easy vector followed by transformation into E. coli strain DH5α, as per manufacturers' instructions (Promega) generating pGPORF (Fig. 3.5). Cloning was confirmed by restriction digestion analysis (Fig. 3.5). PCR was
Fig 3.4. Neucleotide Sequence of General amino acid permease (CaGAP1).
done with the help of Pfu Polymerase (STRATAGENE). The PCR conditions have been given in section 2.6.5.3. For large scale PCR, reactions were set in multiple vials. The PCR products were pooled and were run on a 0.8% preparative agarose gel.

3.2.7. Construction of CaGAP1 expression vector plasmid of S. cerevisiae

3.2.7.1. Cloning of CaGAP1 ORF in expression vector pFL61

A 2224 bp gel purified NotI fragment containing CaGAP1 ORF and downstream portion of the ORF was subcloned into pFL61, a yeast expression vector under PGK promoter generating pFLGP31. The NotI digested 2224 bp band was cut using a sterile blade and eluted by using QIAGEN Min Elute Gel Extraction Kit. 1μl of the eluted band was run on a fresh gel and quantitated visually. The cloning strategy was given in Fig. 3.5.

3.2.7.2. Ligation

Ligation reactions were set up using 50ng of NotI digested pFL61 expression vector and the NotI digested 2224 bp CaGAP1 ORF, at vector: insert ratio of 1:3 nanomoles in 10μl reaction volume containing 1X ligation buffer and 1μl T4 DNA ligase. The reactions were incubated at 16°C for 4 hours.

3.2.7.3. Transformation and screening of recombinants

5μl of the ligation mixes were used to transform DH5α E.coli cells (2.6.6.1). 100μl of the transformation mixes were plated on LB amp plates and incubated at 37°C. The recombinant clones were selected by the migration difference of plasmid in 1% agarose gel. Then 5μg of plasmid DNA of transformed colonies was digested with restriction enzymes (mentioned in Fig 3.5) in a 20μl reaction mix. NEB3 buffer was used. The digestions were incubated at 37°C for 2 hours. The digestions were stopped by heating the reactions at 65°C for 20mins. They were loaded on a 0.8% agarose gel. Orientation of CaGAP1 ORF was also confirmed by restriction digestion.
CaGAP1-ORF PCR amplified from genomic DNA using primer GAPI and GAP2 and cloned into pGEM-T Easy vector.

Restriction digestion analysis of pGPORF

Lane 1 - pGPORF (Uncut)
Lane 2 - 1 Kb ladder
Lane 3 - pGORMEcoRI
Lane 4 - pGORMEcoRV
Lane 5 - pGPORFNotI
Lane 6 - pGPORFPvuII

Restriction digestion analysis of pFLGP31

Lane 1 - pFLGP31 (Uncut)
Lane 2 - 1 Kb ladder
Lane 3 - pFLGP31EcoRV
Lane 4 - pFLGP31NotI
Lane 5 - pFLGP31PvuII

Lane 1- 1 Kb ladder
Lane 2 - gel purified CaGAP1-ORF fragment

Fig 3.5. Schematic representation of the cloning of CaGAPI into Saccharomyces cerevisiae expression vector pFL61.
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

3.2.7.4. DNA Sequence Analysis

To confirm the sequence of CaGAP1 ORF the plasmids pFLGP31 were sequenced by dideoxy chain termination method using the gene specific primer GP1 and GP2, according to the manufacturer’s instructions [Sequenase 2.0 (USB)].

3.2.8. Complementation study of CaGAP1 gene in S. cerevisiae

3.2.8.1. Transformation of expression vector containing CaGAP1 ORF in gap1/gap1 null mutant of S. cerevisiae

Transformation of S. cerevisiae was carried out by the Lithium acetate method as described (Gietz et al., 1992). 5 µg of plasmid pFLPF31 along with denatured 50 µg calf thymus DNA was transformed into the S. cerevisiae gap1 strain MS143. Detailed transformation protocol is described in section 2.6.14.

3.2.8.2. Selection of transformants

The transformation mix was plated on SD-URA medium using URA3 as a selection marker. MS143 (Δgap1) strain was plated as control. The transformants were replica-plated on SD medium containing suitable amount of supplements without uridine. Ura positive transformants (Δgap1:CaGAP1) were tested on minimal proline plates containing 20 µg uridine ml⁻¹ and 75 µg mimosine ml⁻¹ (Fig 3.6).

3.2.9. Assay of amino acid uptake

S. cerevisiae and C. albicans strains to be assayed were cultured in SD medium to A₆₀₀~2.0. Cells were collected by filtration on 0.45 µm nitrocellulose filter (Sartorius AG) and resuspended in SPD medium. The [¹⁴C] citrulline was added to exponentially growing cultures. Samples of 0.5 ml were removed periodically after each 2.5 min; rapidly collected by filtration through glass fiber filter (Whatman, Cat No-1822 025) and washed with chilled water. Filters were dried under heat lamp and placed in 5 ml toluene based liquid scintillation cocktail. The counts were taken in Wallac DSA-based liquid scintillation counter. The specific activity of [¹⁴C] citrulline used was 2.1GBq/mmol. Labeled citrulline was obtained from Perkin Elmer Life Sciences.
Fig. 3.6. Complementation studies of general amino acid permease of *C. albicans* in *S. cerevisiae*. Growth of mutant strain, MS143 (Δ*gap1*) of *S. cerevisiae* and Transformants ((Δ*gap1*:CaGAP1)) on various media as indicated. The mutant strain was *ura3* but otherwise prototrophic. Different media are described in Materials and Methods (section 3.2.2).
3.2.10. GlcNAc Induction studies of CaGAPI

3.2.10.1. Induction of CaGAPI at different time points

*Candida albicans* SC5314 cells were precultured in GPK medium and resuspended in 100X volume of fresh GPK. Cultures were grown to A_600 ~ 2.0. Harvested cells were washed twice with 0.3% KH_2PO_4, resuspended in an equal volume of NPK, and incubated at 30 °C. The treated cells were harvested at different time points of growth as described in the Results section and frozen at -20 °C until use. Control cells were resuspended in GPK instead of NPK.

3.2.10.2. Effect of nitrogen sources in CaGAPI induction

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, SGN with 2 % GlcNAc and grown for 2 hours 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. Similarly 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, HLC67 were grown similarly in SN media with 2 % GlcNAc for 2 hours at 30 °C and control cells were cultured in SC with 2% dextrose.

3.2.10.3. RNA extraction and Northern Analysis

Total RNA was extracted from frozen cells (Ausubel *et al.*, 1994), and electrophoresis on 1.5% formaldehyde agarose gel was carried out with 40 µg of RNA per lane, and subsequent Northern blot analysis was performed as described (Ausubel *et al.*, 1994) with a ^32_P-labeled 938 bp EcoRV fragment of CaGAPI (Fig. 3. 7C), excised from pCaGAPI. Details of Northern analysis were described in section 2.6.10, 2.6.11 and 2.6.12.

3.2.11. CaGAPI Gene disruption in *C. albicans*

The “URA-blaster” strategy was used to make a null mutant of CaGAPI in *C. albicans* (Fonzi and Irwin, 1993).
3. 2 11. 1. Construction of disruption cassette

The entire 3512 bp CaGAP1 fragment was PCR amplified from genomic DNA of C. albicans SC5314 using oligonucleotides 5' CATTACCTGGTGCCACTCC3' and 5' GGTTTCGAATCAGTCGATGG3' as primers and cloned in pGEMT-Easy vector followed by transformation into DH5α to generate pCaGAP1(Fig. 3. 7). To obtain Δcagap1 mutants, plasmid pGPI was constructed by replacing a 948 bp EcoRV-EcoRV fragment of pCaGAP1 containing the CaGAP1 ORF with a 4176 bp blunt ended ScaI-Pvull fragment of vector pUC19-CUB containing the hisG-URA3-hisG cassette (Fig. 3. 10 and Fig. 3. 11). To obtain pUC19-CUB the 4 kb BamHI-BgII hisG-URA3-hisG cassette from pCUB6 was integrated into the BamHI site of plasmid pUC19 (Fig. 3. 8 and Fig 3. 9). Detailed strategy of construction of disruption cassette is described in Fig. 3. 7 to 3. 12.

3. 2 11. 2. First allele disruption of CaGAP1 in C. albicans strain CAF3-1

The ura minus strain of wild type C. albicans, CAF3-1 was kindly provided by W.A. Fonzi. For the first allele disruption 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 pGPI (Fig. 3. 12 and 3. 18A). The transformation mix was plated in SD plates and incubated at 30 °C for 2 days. As a negative control of transformation, water was used instead of the digested DNA. The Ura positive transformants obtained after 2 days were inoculated in 10ml YPD and genomic DNA was isolated from them (section 2. 6. 14). Around 5μg of genomic DNA was digested in 20μl reaction volume with AatII and SalI enzymes. The reactions were incubated at 37 °C for overnight. The digested DNA samples were loaded on a 0.8% agarose gel along with a 1kb ladder (Fig 3. 14). After electrophoresis, a tracing of the gel, and photograph, were taken to mark the positions of the bands. The gel was further processed for Southern blot analysis. The digested DNA were transferred to a nylon membrane, fixed and probed with 3.5 kb NotI fragment of pCaGAP1. The positive clones having his-URA3-his insertion in one of the two alleles were selected for further analysis (Fig. 3. 14). The resultant first allele disruptants were cured of the URA3 marker for second round of transformation.
CaGAP1 gene amplified from genomic DNA using primers G1 and G2

Fig. 3. Cloning of CaGAPI gene of *Candida albicans* in pGEM-T easy vector.

A. 3.5 Kb PCR fragment run on 8% agarose gel.

B. 3.5 Kb gel purified CaGAPI fragment run on 8% agarose gel with 1 Kb Ladder.

C. Restriction Digestion analysis of pCaGAPI clone.
The 31 bp and 346 bp represent parts of derivative plasmids.

Digested with BamHI, Bgl II and Pvu II

Ligated and screened for transformants

Fig. 3.8. Schematic representation of construction of pUC19-CUB. 4 Kb fragment of pCUB6 is cloned into high copy number plasmid pUC19 to obtain the cassette for disruption of CaGAP1 easily.
Fig. 3.9. Restriction digestion analysis in cloning of pUC19-CUB

A. 1 µg of DNA of pCUB6 digested with BamHI, BglII and PvuII. The 4 Kb BamHI-BglII band contains $\text{hisG-URA3-hisG}$ cassette. Digestion with PvuII was to get rid of the overlapping 4 Kb fragment of vector backbone, which break into 2.3 Kb and 1.7 Kb band. Digest was run on 0.8% agarose gel with 1 Kb Ladder.

B. 1 µg of DNA of correct transformants(pUC19-CUB) digest with AatII, BsiHKaI and BstXI. Digests were run on 0.8% agarose gels with 1 Kb DNA Ladder as a marker. There is no BstXI site in the disruption cassette and pUC19 vector backbone.
Digested with SacI and PvuII, releasing 945 bp fragment
End filled and ligated

pGP1 (5579bp+4175bp)

Digested with Eco RV releasing 945 bp fragment

pCaGAPI (5579bp)

Fig. 3.10. Schematic Diagram showing construction of the cassette for Disruption CaGAPI
Fig. 3.11. Restriction digestion analysis in construction of CaGAPI disruption cassette

A. 1 μg DNA of pUC19-CUB digested with SacI and PvuII, showed bands of 4.176 Kb that house with his-G-URA3-his-G cassette, and the 2.460 Kb pUC19 backbone.
B. 2 μg DNA of pCaGAPI digested with EcoRV, showed bands of 5.681 Kb and 0.948 Kb.
C. Higher migrating clones were selected to screen for recombinants. Correct recombinants is masked with asterisks.
D. Restriction Digestion analysis of pGP1.
Fig. 3.12 Transformation fragment of cagap1: his-G-URA3-his-G : cagap1 cassette.

Digested with NotI to release the 6.688 Kb cassette of cagap1: his-G-URA3-his-G : cagap1 along with pGEM-T Easy vector backbone.
A. *C. albicans* transform with the 6.688 \textit{NcoI} fragment pGP1

B. Homologous recombination and excision of \textit{URA3} with one \textit{hisG} sequence.

C. Selection on 5-FOA for \textit{ura3} cells. Transformation and selection on 5-FOA repeated for disruption of the second allele

D. Both alleles disrupted, one \textit{hisG} sequence remain.

Fig. 3.13. Schematic Diagram Showing Disruption of \textit{CaGAPI} by "Ura-blaster" Technique
3. 2. 11. 3. Curing of URA3 Marker

Two of the first allele disruptants (GP5) were streaked on YNB-dextrose-uridine-FOA plate (2. 6. 2) for single colonies, and grown at 30°C for 2 days. The single colonies (GP57) were patched simultaneously on SD and SD-uridine plates, and grown at 30 °C for a day. The strains growing in SD-uridine plates only were inoculated in 10 ml of YPD supplemented with uridine. The cultures were grown till saturation. Genomic DNA was isolated and Southern (Fig. 3. 14) was done exactly as described above.

3. 2. 11. 4. Disruption of Second Alleles

One Ura cured transformant (GP57) was then used to delete the second allele of CaGAP1 by using a similar process to generate the homologous gapI/gapI mutants GP573 (ura+) and GP5731 (ura-) (Fig. 3. 14C and 3. 18C).

3. 2. 12. Construction of CaGAP1 revertant strain, GP57315 in C. albicans

3. 2. 12. 1. Preparation of CaGAP1 revertant construct

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 pCaGAPI located downstream of CaGAP1 ORF. Detailed strategy of preparation of revertant construct is given in Fig. 3. 15. to 3. 17.

3. 2. 12. 2. Construction of revertant strain

The homozygous mutant GP5731 (ura-) was then transformed with a 5.8 kb NotI fragment derived from pGP2 (Fig. 3. 18B). Transformants were selected on SD minimal medium to obtain Ura+ strain, which was confirmed by southern analysis (Fig 3. 18C).

3. 2. 12. 3. Southern Analysis

For screening of mutants and revertant strains, 5 μg of genomic DNA from each transformant and parent strain were digested with AatII and SacI, electrophoresed and transferred (Sambrook et al.,
Fig. 3.14. Schematic representation of first and second allele disruption of CaGAPI gene of C. albicans
Fig. 3. 15. Schematic Diagram Showing the Strategy for Reintroduction of One copy of CaGAP1 into the genome of the Null Mutant.
Fig. 3.16. Restriction digestion analysis in construction of *CaGAPI* revertant cassette

A. 2 µg DNA of pCaGAPI partially digested with *Bst*XI showed 6.5 kb linear fragment. 2µg DNA of pUC19-CUB digested with *Eco*RV showed bands of 2.3 Kb that house with *URA3* gene plus some portion of *hisG*, 3.9 Kb and 0.5 kb pUC19-CUB backbone.

B. 6.5 Kb (as Vector) and 2.3(as insert) Kb gel purified fragment run on 0.8% agarose gel with 1 Kb Ladder.
Fig. 3. 17. Restriction digestion analysis pGP2 (Revertant construct)

A. Higher migrating clones were selected to screen for recombinants. Correct recombinants is marked with asterisks.

B. Restriction Digestion analysis of pGP2.
Fig. 3. 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-), GP-5(+/Δcagap1 ura+), GP57 (+/Δcagap1 ura-), GP573 (Δcagap1/Δcagap1 ura+), GP5731 (Δcagap1/Δcagap1 ura-) and a revertant strain GP57315 (Δcagap1/Δcagap1+CaGAP1 ura+) 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. Exact size and genotype of the expected hybridizing DNA fragment are indicated on the right.
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

1989) to Genescreen Plus membrane (NEN Research Product). The blots were hybridized with a $^{32}$P labelled 3.5 kb NotI-NotI fragment from pCaGAPI (Fig. 3. 18C).

3. 2. 13. Induction of filamentation by Serum and GlcNAc

*Candida* cells were grown to the logarithmic growth phase in YPD, washed twice with sterile water and shook for 10 hours in water at 30°C at 100 rpm (Sonneborn *et al.*, 2000). Cells ($A_{600}$=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 hours or with Calf serum bovine (SIGMA Cat # C-6278) in YPD at 37°C for 2 hours.

3. 2. 14. Morphogenesis studies in solid media

*Candida* strains were grown in SD at 30°C, counted using a haemocytometer, and plated at a conc. of 80-100 cells per spider (1 % nutrient broth, 1 % mannitol, 0.2 % K$_2$HPO$_4$, 2 % bacto-agar) or SLAD (0.17 % YNB without amino acid and ammonium sulphate, 2 % dextrose, 50 μM ammonium sulphate, 2 % bacto-agar) plate. Plates were incubated at both 30°C and 37°C for 7-10 days.

3. 2. 15. Determination of virulence (Murine Model)

Five to six weeks old female swiss mice were intravenously injected with $10^6$ 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. Groups were made based on strains, and were kept in separate cages. Observations were recorded once in 24 hours for morbidity. Animals were treated as per guided rules followed in India.

3. 3 RESULTS

3. 3. 1. Sequence analysis of the CaGAP1 gene in Candida albicans

Sequencing of *C. albicans* CaGAP1 gene followed by BLAST search revealed that it is homologous to GAP1, the general amino acid permease of *S. cerevisiae* (Jauniaux and Grenson, 1990). The sequence was submitted to the EMBL databank and assigned accession no AF467941.
LOCUS AF467941 1749 bp DNA linear PLN 07-FEB-2002
DEFINITION Candida albicans Gap1 protein gene, complete cds.
ACCESSION AF467941
VERSION AF467941.1 GI:18568389
KEYWORDS Candida albicans.
ORGANISM Candida albicans
Eukaryota; Fungi; Ascomycota; Saccharomycotina; Saccharomycetes;
Saccharomycetales; mitosporic Saccharomycetales; Candida.
REFERENCE 1 (bases 1 to 1749)
AUTHORS Biswas, S., Roy, M. and Datta, A.
TITLE CAGAPI gene of Candida albicans
JOURNAL published
REFERENCE 2 (bases 1 to 1749)
AUTHORS Biswas, S., Roy, M. and Datta, A.
TITLE Direct Submission
JOURNAL Submitted (12-JAN-2002) School of Life Sciences, JNU, New Delhi, Delhi 110067, India
FEATURES Location/Qualifiers
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DFFLGYFVPLASWIGYKIKWIRDLWTLFIRAKDIDLDTGRINVDLDLLQQEIAEKEA
QLAERPYYRIFRFC"

Fig. 3. 19. Sequence of General amino acid permease (CaGAPI) gene product of C. albicans. The EMBL accession number for the sequences reported in this chapter is AF467941.
Cloning, complementation, expression and mutation studies of CaCAP gene in Candida albicans (Fig. 3. 19). The sequence contained a single open reading frame of 1746 nucleotides, which encodes a predicted protein of 582 amino acids with an estimated Mr of 63950.

3. 3. 2. Promoter analysis of CaGAPI gene

Several putative TATA box sequences along with a global regulator Gcn4p and AP-I binding site appear at position -286 and -152 respectively upstream from the initiation codon ATG. Interestingly there is one 5'GAATAG 3'sequence (at -646 position) and several TTGTT/TGGTT sequences were found upstream of CaGAPI promoter. GAATAG /GATA type sequences which are binding target of transcription factor Gln3p were also found in GAPI promoter of S. cerevisiae (Miller & Magasanik, 1991). TTGTT or TGGTT plays an auxiliary role in activation of nitrogen regulated gene by Gln3p (Stanbrough et al., 1996). Another transcription factor Cph1p/Acprp of Candida albicans, homolog of Ste12p of S. cerevisiae binds to a heptamer sequence TGAAACA, referred to as pheromone responsive element (PRE). This sequence is also present at -989 position of CaGAPI promoter (Fig. 3. 20).

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 CaGAPI bears a marked resemblance to some previously sequenced yeast permease genes like HIP1 (Histidine permease), TAT2 (Tryptophan permease), AGP1 (Arginine/Glutamate permease) etc. with overall sequence similarity of 40-50% (Fig. 3. 21).

3. 3. 3. Hydropathy profile

The protein product (AAL76065.1) that was deduced from the CaGAPI gene sequence is considerably hydrophobic containing 46 % non-polar residues. A hydropathy profile generated with Kyte and Doolittle's algorithm (1982) showed that there are 10-12 transmembrane regions within the protein (Fig. 3. 22A). Hydrophobic segments of at least 20 amino acids were revealed with an average hydropathy value lower than 1.3, suggesting the formation of membrane spanning α helices by these segments (Lodish, 1988). These transmembrane regions are interconnected with hydrophilic regions that frequently contain cluster of positively or negatively charged amino acids (Fig. 3. 22B). The N-terminus of the CaGAPI 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 server of the Swiss Institute of Bioinformatics.
Fig. 3. 20. The CaGAP1 promoter sequence. Nucleotides are numbered on right from the ATG initiation codon of CaGAP1 open reading frame (position +1). GAATAG site, PRE binding site, GAATAG site, TTGT sites, TTGTT sites are boxed. Gcn4p binding site and AP-1 binding site are underlined.
Fig. 3. 21. Sequence similarity of CaGAP1 (AF467941) of C. albicans with GAP1 general amino acid permease (CAA82113). ORF YKR039w), HIPI histidine permease (CAA97217. ORF YGR191w) and TAT2 tryptophan permease (CAA99020). ORF YOL020w) of S. cerevisiae. Alignments were obtained by the CLUSTAL program and the log odds matrix of Dayhoff to evaluate conservative replacements. Dashes indicate gaps introduced to optimize the alignment; (*) indicate identical amino acid residues; (; & ) indicate conservative replacements.
Fig. 3. 22. **Hydropathy plot.** (A) Hydrophobicity and hydrophilicity values (respectively above and below the horizontal line) deduced with the algorithm of Kyte and Doolittle (1982), using 20 amino acid window. (B) Distribution of Non-polar, charged, positively and negatively charged residues respectively.
3.3.4. *CaGAP1* gene present as a single copy number in genome

There is one gene in *S. cerevisiae* encoding general amino acid permease (*GAP1*). Genetic relatedness between *S. cerevisiae* and *C. albicans* made it probable that *C. albicans* would also have one gene for *CaGAP1*. Hybridization experiment was carried out (Southern blot) with total genomic DNA from *C. albicans* strain SC5314. Genomic DNA was digested with 5 restriction enzymes, transferred to a nylon membrane and probed with the 948 bp *EcoRV* fragment of *CaGAP1* (Fig. 3. 23A). One hybridizing band was obtained with enzymes which did not cut inside the probe and two with those which did, although an additional unexplained (may be due to hybridization problem) band was also visible in the *NsiI* digest. (Fig. 3. 23B)

3.3.5. *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 acid and related compounds, such as ornithine and citrulline, and several D-amino acids and toxic amino acids analogs 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 (MSI38) of *gap1* mutant (MSI43) of *S. cerevisiae* and the transformants (*Δgap1::CaGAP1*) failed to grow on minimal proline media (SPD) containing mimosine whereas *gap1* mutant, MSI43 was not sensitive to mimosine (Fig. 3. 24A, C and Fig. 3. 6), indicating the functional similarity of Cagap1p with Gap1p of *S. cerevisiae*. The failure of the transformants (*Δgap1::CaGAP1*) to grow on minimal proline 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. 3. 24 E) and GlcNAc containing minimal proline media, SPN (Fig. 3. 24F) when mimosine was added. But the effect of mimosine in *C. albicans* persists only upto a maximum of 10-15 hours as a result of which we found no significant difference in growth on solid plates after 2 days (Fig. 3. 24G, H). This may be due to the higher growth rate of *C. albicans* as compared to *S. cerevisiae*.

3.3.6. Effect of nitrogen source on the amino acid analog resistant phenotypes

Amino acids are transported into *S. cerevisiae* both specific and non specific transport systems. General amino acid permease system is strongly repressed when growth medium contains
Fig. 3.23. CaGAP1 is a single copy gene. (A) Region from CaGAP1 used as probe. (B) Southern blot analysis suggested that CaGAP1 is a single copy gene. Genomic DNA from C. albicans from SC5314 digested with EcoRI, EcoRV, NsI, Pvull, XmnI. Southern blot analysis was performed using high stringency conditions.
Fig. 3. 24. Phenotype of general amino acid mutant strain of S. cerevisiae and C. albicans. S. cerevisiae strain MS138 (wild-type), MS143 (Δgap1/Δ) and MSPF31 (Δgap1/Δ::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. S. cerevisiae strain MS138, MS143 and MSPF31 grown on solid SPD (C) and SAD (D) at 30 °C for 2 days. C. albicans strain SC5314 (wild-type), GP573 (Δcagap1/Δ) and GP57315 (Δcagap1/Δ+CAGAPI) incubated in liquid glucose containing minimal proline medium SPD (E) and GlcNAc containing minimal proline medium SPN (F) at 30 °C for indicated time period. C. albicans strain SC5314, GP573 and GP57315 grown in solid glucose containing minimal proline medium SPD (G) and GlcNAc containing minimal proline medium SPN (H) at 30 °C for 2 days.
Cloning, complementation, expression and mutation studies of \( \text{CaGAP} \) gene in \( \text{Candida albicans} \) (\( \text{NH}_4 \)\(_2\)\( \text{SO}_4 \) and glutamate (Springael \& Andre, 1998). To investigate such an effect on the regulation of Cagaplp we did growth kinetics as well as replica plating of wild-type strain MS138, the mutant MS143 (\( \Delta \text{gap1} \)) and transformant (\( \Delta \text{gap1}:\text{CaGAP1} \)) of \( S. \text{cerevisiae} \) on media containing ammonia (SAD) and glutamate (SED) as nitrogen source in the presence of mimosine. Interestingly, wild type strain and transformants were found to be resistant to mimosine in SAD (Fig. 3. 24B, D and Fig. 3. 6) and SED (Fig. 3. 6). These results suggested that in ammonia and glutamate containing medium, mimosine uptake is lowered due to inactive general amino acid permease system. Moreover, we observed a similar effect when we did growth kinetics as well as 2 days inoculation on solid plates of the \( C. \text{albicans} \) 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).

3.3.7. \( \text{CaGAP1} \) mutation affects Citrulline uptake

To explore whether the Cagaplp of \( C. \text{albicans} \) allows uptake of amino acid and related compounds, citrulline uptake assay was performed in minimal proline medium (SPD), to demonstrate the general amino acid permease activity in \( S. \text{cerevisiae} \) as well as in \( C. \text{albicans} \). In \( cagap1 \) null mutant (GP573) the citrulline uptake rate was 2 times lower than the wild type strain (SC5314) and a revertant strain (GP57315) in \( C. \text{albicans} \) (Fig. 3. 25B). Interestingly, the citrulline uptake of transformants (\( \Delta \text{gap1}:\text{CaGAPI} \)) was increased to 2.5 fold than the \( \text{gap1} \) mutant of \( S. \text{cerevisiae} \) (Fig. 3. 25A). This indicates the functional similarity of general amino acid permease of the two microorganisms.

3.3.8. Effect of different nitrogen sources on GlcNAc induction of \( \text{CaGAPI} \)

The \( \text{CaGAPI} \) 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 \( \text{CaGAPI} \) in glucose-grown and GlcNAc-grown cultures at various intervals. A significant induction was observed in GlcNAc-grown cells at 2 hours of growth (Fig. 3. 26B).

Northern blot analysis was also used to investigate the effect of different nitrogen sources upon GlcNAc induction of \( \text{CaGAPI} \) (Fig. 3. 26C). Intensity of the individual band was quantified by densitometry of the autoradiogram and fold induction has been represented graphically in Fig. 3. 26D. It was observed that in SEN (glutamate), SPN (proline), SUN (urea) or SGN (glutamine)
Fig. 3.25. General amino acid permease activity of *S. cerevisiae* and *C. albicans*. *S. cerevisiae* strain MS138 (wild-type), MS143 (Δgap1/Δ) and MSPF31 (Δgap1/Δ:CaGAP1) (A) and *C. albicans* strain SC5314 (wild-type), GP573 (Δcagap1/Δ) and GP57315 (Δcagap1/Δ+CAGAPI) (B) were grown exponentially on SD media in 30°C. Cells were harvested by filtration and resuspended in minimal proline (SPD) medium and [14 C] citrulline were added to a final concentration of 4 μM. 0.5 ml aliquot was taken out at 30 sec interval over a period of 2.5 minutes. Imported amino acid was detected as total counts associated with washed cell bodies. Relative rate of [14 C] citrulline uptake are expressed as a percentage of wild-type uptake.
Cloning, complementation, expression and mutation studies of CaGAPI gene in Candida albicans

media, the level of CaGAPI mRNA was about 1.4 fold higher than that of control cells grown in only GlcNAc containing medium (SN) whereas CaGAPI 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 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).

3.3.9. Expression of CaGAPI is regulated by Cph1p mediated Ras1p Signaling but is independent of Efg1p

To investigate the effect of different mutations on the expression of GlcNAc inducible CaGAPI, the following strains N-2-1-6 (Δdac1Δnag1Δhxk1/Δdac1Δnag1Δhxk1), N-2-1-6+p33 (Δdac1Δnag1Δhxk1/DAC1NAG1Δhxk1), A-11-1-1-4 (Δacpr1/Δacpr1), CAN52 (Δras1/Δras1), HLC67 (Δefg1/Δefg1) and AS1 (Δtpk2/Δtpk2) were used. Northern blots showed transcript levels of CaGAPI mRNA declined in case of Δras1/Δras1 and Δacpr1/Δacpr1 null mutant and remained unaffected in N-2-1-6, N-2-1-6+p33, HLC67, AS1 strains (Fig. 3. 26E). This implies that Acpr1p/Cph1p mediated Ras1p signaling regulates the CaGAPI whereas cAMP dependent protein kinase A and Efg1p mediated Ras1p signaling pathway is not involved in CaGAPI expression. Although DAC1, NAG1 and HXK1 are induced by GlcNAc but these GlcNAc catabolic pathway genes are not involved in CaGAPI expression.

3.3.10. Physiological Effects of Disruption of CaGAPI gene

To determine the role of CaGAPI in the physiology of C. albicans, we disrupted both chromosom al copies of the gene sequentially by URA blaster technique (Fonzi & Irwin, 1993). Growth rates of 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). All Ura+ strains were used for growth kinetics and morphological studies.

3.3.10.1. Effect of CaGAPI null mutation on virulence

Much recent research on the pathogenesis of disseminated C. albicans infection has focused on genes encoding putative virulence factors. The criterion for virulence is usually measurement of survival times of intravenously infected mice (Csank et al., 1998; Yamada-Okabe et al., 1999). In
Fig. 3. 26. Differential expression of CaGAP1. (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 hours at 30 °C. A northern blot of the sample was hybridized with 938 bp EcoRV fragment of pCaGAP1. Each lane contains 40 μg of RNA. (C) Effect of GlcNAc induction in different nitrogen source. Total RNA was isolated from SC5314 cells in presence of GlcNAc (2 %) in different synthetic media, SN, SEN, SPN, SAN, SUN and SGN as described in Materials and Methods and examined by Northern blot analysis. The same gene probe used here and each lane contained 40 μg of RNA. (D) CaGAP1 expression levels in media containing alternate nitrogen sources at 2 hour time point were quantified by densitometry of autoradiogram and fold induction was represented graphically in respect of synthetic complete GlcNAc medium (SN) (E) Effect of GlcNAc induction in different mutants of C. albicans. Total RNA was isolated from different strain of C. albicans, SC5314, N-2-1-6, N-2-1-6+p33, CAN52, HLC67, AS1and A-11-1-1-4 in SC media in presence of GlcNAc (2 %) as described in Materials and Methods and examined by Northern blot analysis.
order to investigate whether GlcNAc inducible CaGAP/I plays any role in conferring virulence to this organism hematogenously disseminated candidiasis of wild-type and CaGAP/I mutant strains, was studied in murine model. But no change in virulence was observed with the mutant strains of CaGAP/I gene (Fig. 3. 27).

3. 3. 10.2. Effect of CaGAP/I disruption on germ tube inducing liquid media

Serum is still the magic potion to rapidly induce true hyphae in C. albicans, though the factor responsible for induction is yet to be identified. It is not albumin, since albumin-free serum from a rat mutant was as efficient at promoting hyphae as normal serum (Feng et al., 1999). GlcNAc and proline may contribute to the serum effect, since they are generated by degradation of serum (glycol-) proteins (reviewed by Ernst, 2000). The yeast cells start producing germ-tubes on induction with GlcNAc at 37°C (Shepherd et al., 1980). So we wanted to check if the cagapJ/cagapJ mutants still retained the ability to undergo dimorphic transition with GlcNAc and serum as the inducer. When GlcNAc was used as a carbon source, growth rate was higher but no striking difference was found among the wild-type and mutant strains of CaGAP/I gene. C. albicans can shift from yeast to a hyphal form when it is cultured at 37°C in presence of serum and GlcNAc after 2 hour. This transition was not impaired or affected in a cagapJ/cagapJ mutant (GP573) in both serum (Fig. 3. 28A, B, C, D) and GlcNAc induction media (Fig. 3. 28E, F, G, H). However, we observed less hyphal clump formation by GlcNAc in cagapJ/cagapJ mutant in shake flask (Fig. 3. 28G). No difference was found in heterozygous mutant and heterozygous revertant with respect to this behavior (Fig. 3. 28F, H).

3. 3. 10.3. Effect of CaGAP/I mutation on filament inducing solid media

Then we 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 cagapJ null mutant (GP573) showed less hyphal formation and altered colony morphology which was different from wild type strain and heterozygous mutant (Fig. 3. 29 I, J, K, Q, R, S). This phenotype was reversed by reconstituting a single functional copy of the gene (Fig. 3. 29L, T). An interesting feature of our analysis was the finding that both heterozygous and homozygous mutant had an obvious defect in filamentation and drastic abnormal colony morphology in nitrogen starvation condition i.e on solid SLAD plates at 37°C (Fig. 3. 29 N, O). Furthermore, the defect in filamentation and colony morphology is not fully suppressed by introduction of a single copy of
Fig. 3. 27. Effect of ΔCagap1 mutations on virulence in disseminated infection. Mice (n=8) were injected intravenously with 1X 10^6 cells of strain SC5314 (Wild-type), GP573 (Homozygous mutant) and GP57315 (Heterozygous revertant). The number of surviving mice was recorded each day.

Fig. 3. 28. Morphology of CaGAP1 mutant strains under hypha-inducing liquid conditions. Wild type SC5314 (CaGAP1/CaGAP1), heterozygous mutant, GP5 (Δcagap1/CaGAP1), homozygous mutant, GP573 (Δcagap1/Δcagap1), Heterozygous revertant GP57315 (Δcagap1/Δcagap1 strain in which CaGAP1::URA3 has been recombined back at the CaGAP1 locus, Δcagap1/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 μm) but shows less hyphal clump in GlcNAc inducing condition (E, F, G, H) (Bar, 100 μm)
Fig. 3. 29. Morphology of *CaGAP 1* mutant strains under solid hypha-inducing media. Wild type SC5314 (*CaGAP 1/CaGAP 1*), heterozygous mutant, GP5 (*Δcagap 1/CaGAP 1*), homozygous mutant, GP573 (*Δcagap 1/Δcagap 1*), Heterozygous revertant (*Δcagap 1/Δcagap 1* strain in which *CaGAP 1::URA3* has been recombined back at the *CaGAP 1* locus, *Δcagap 1/CaGAP 1::URA3*), were induced for filamentation under different conditions. *cagap 1/cagap 1* null mutant showed defective filamentation on solid Spider (I, J, K, L) (Bar, 2mm) and SLAD medium Nitrogen starvation (SLAD medium) (M, N, O, P) (Bar, 1mm) also affects the filamentation and colony morphology in case of heterozygous mutant and revertant strain of *CaGAP 1*. Colonial rim appearance of the above mentioned strains in solid spider plates (Q, R, S, T)
Cloning, complementation, expression and mutation studies of \( \text{CaGAPI} \) gene in \( \text{Candida albicans} \)

Functional gene (Fig. 3. 29 P). However, \( \text{Cagap1/Cagap1} \) homozygous disruptants were more homogeneous than heterozygous strain and showed a greater reduction in peripheral hyphal growth indicating that gene dosage is important for morphogenesis of \( \text{C. albicans} \) under certain conditions.

3. 4. DISCUSSION

We have isolated the general amino acid permease gene, \( \text{CaGAPI} \) from \( \text{C. albicans} \), 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 \( \text{C. albicans} \). The results presented here are in agreement with three essential points: first, the activity of a general amino acid permease was regained when a \( \text{gap1} \) mutant strain of \( \text{S. cerevisiae} \) was transformed with the \( \text{CaGAPI} \) gene in minimal proline media indicating the functional similarity of \( \text{Cagaplp} \) with \( \text{Gaplp} \), second, the transcription of \( \text{CaGAPI} \) is regulated by external nitrogen source and is dependent on \( \text{Cphlp} \) mediated \( \text{Rasl p} \) signaling, and finally defective filamentation or abnormal colony morphology in homozygous and heterozygous \( \text{CaGAPI} \) disruptants were found under certain conditions.

\( \text{CaGAPI} \) (AF467941) is not only homologous to \( \text{GAP1} \) (CAA82113) of \( \text{S. cerevisiae} \) but also shows similarity to other yeast permease genes such as \( \text{HIP} \), \( \text{TAT2} \), \( \text{AGP1} \), \( \text{GPN1} \), (Jauniaux and Grenson, 1990). The deduced gene product is highly hydrophobic with 10-12 transmembrane regions. \( \text{CaGAPI} \) was induced by GlcNAc at 2 hours of growth but was expressed only at a basal level in Glucose containing complete medium. The GlcNAc induction of \( \text{CaGAPI} \) was enhanced in synthetic minimal media supplemented with a single amino acid like glutamate, proline, glutamine or urea and but was inhibited by ammonia. The regulation of \( \text{CaGAPI} \) at the level of transcription is comparable to \( \text{GAP1} \) regulation in yeast, where the transcription factors \( \text{Gln3p} \) (in presence of glutamate) and \( \text{Nil1p} \) (in presence of urea or proline) are activators (Stanbrough et al., 1995), while \( \text{Dal80p} \) (Cunningham and Cooper, 1993) and \( \text{Nil2p} \) (Lodish, 1988; Rowen et al., 1997) are inhibitors. In the presence of ammonium, Ure2p, another transcriptional repressor sterically hinders \( \text{Gln3p} \) from activating \( \text{GAP1} \) (Blinder et al., 1996). These factors bind to an upstream regulatory sequence containing a motif surrounding a core GATA sequence (Springael and Andre, 1998). The obvious similarity between the \( \text{CaGAPI} \) promoter and nitrogen regulated gene promoter like \( \text{GAP1} \), \( \text{GLN1} \), \( \text{GDH2} \) etc. of \( \text{S. cerevisiae} \) is the presence of GAATAG sequence (Cunningham and Cooper, 1993). Another feature common to the \( \text{CaGAPI} \) and \( \text{GAP1} \) promoter is the presence of TTGTT or TTGTT, which plays an auxiliary note in activation by \( \text{Gln3p} \) (Miller
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

and Magasanik, 1991). Five GATA-type transcription factors and one gene homologous to URE2 have been reported from Candida albicans Genome Sequencing Project, Stanford.

Very recently Fonzi’s group demonstrated that induction of CaGAP1 in the presence of proline was GAT1-transcription factor dependent. Deletion of a single copy of GAT1 had no discernable effect on the pattern or level of expression of CaGAP1 gene but double allele knockout of GAT1 gene causes drastic reduction of CaGAP1 expression (Limjidaporn et al., 2003). Candida albicans GAT1 was identified based on its homology with other fungal GATA factors involved in nitrogen regulation. As expected of a nitrogen regulatory factor, a null mutation in GAT1 altered the nitrogen utilization capacity of the cells. Growth of gat1 mutants was normal with a number of nitrogen sources, but was compromised on isoleucine, tyrosine and tryptophan. The modest phenotype of the gat1 mutant may indicate redundant uptake or utilization pathways or overlapping transcriptional control as seen for GLN3 and GAT1/NIL1 of S. cerevisiae (Coffman et al., 1997). Mutation of GAT1 or GLN3 individually reduces GAP1 expression to approximately 50% while expression is completely eliminated in the double mutant (Stanbrough et al., 1995; Coffman et al., 1997). Even GAT1 has been identified not only as a mediator of nitrogen regulation in C. albicans. It plays a major role in virulence of C. albicans.

In our induction studies we also saw that CaGAP1 is GlcNAc inducible but in the GlcNAc catabolic pathway mutants dac1nag1hxk1/dac1nag1hxk1 (Nag regulon mutated) and dac1nag1hxk1/DAC1NAG1hxk1 (Hexokinase mutant), which is incapable of utilizing GlcNAc (Singh et al., 2001), there is 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 NH2-terminal region of Acprlp/Cphlp can recognize and bind pheromone responsive elements (PRE) in vitro like Ste12p of S. cerevisiae (Malathi et al., 1994). Interestingly one PRE sequence TGAAACA is also present in CaGAP1 promoter. This clearly showed the role of Cph1p dependent Ras1p signaling in GlcNAc induced CaGAP1 expression (Fig. 3. 30).

Gap1p of S. cerevisiae is not only regulated transcriptionally but its activity also depends on external nitrogen source. Addition of ammonium ions (Springael and Andre, 1998; Bernard and
Cloning, complementation, expression and mutation studies of CaGAP1 gene in Candida albicans

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 transformant (Δgap1::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 persist a maximum of 10-15 hours, which may be because of higher growth rate of this microorganism.

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 [14C] citrulline (Grenson et al., 1970). To demonstrate the import of amino acids by Cagap1p, citrulline uptake assay was performed in minimal proline medium. General amino acid activity was increased 2.5 fold when CaGAP1 gene was expressed in gap1 mutant strain (Δgap1::CaGAP1) of S. cerevisiae. On the other hand cagap1 mutant (GP573) of C. albicans showed 50% less citrulline uptake than wild-type strain (SC5314) and the permease activity regained when CaGAP1 gene was recombined back in CaGAP1 locus of cagap1 mutant strain (GP57315). So we could not exclude the possibility that transport pattern of general amino acid permease is same in both S. cerevisiae and C. albicans.

Here we have also shown that cagap1/cagap1 have 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 in GlcNAc inducing condition. Defective morphology and less filamentation of both the heterozygous and homozygous mutant in nitrogen starvation strongly suggest that the GlcNAc inducible CaGAP1 is regulated by external nitrogen source. Thus one interpretation of this 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
Fig. 3. 30. Hypothetical model of regulation of general amino acid permease expression and its activity in respect of different environmental conditions. In poor nitrogen sources like minimal proline medium or under nitrogen starvation conditions CaGAP1 is induced by GATA factor, GATI. In rich nitrogen sources like ammonia CaGap1p sorted from plasma membrane and degraded by ubiquitin-triggered internalization. CaGAP1 is also induced GlcNAc through Cph1p-mediated Ras1p signaling which leads to a morphological changes. Neutrophil induce the expression of general amino acid permease. The general amino acid permease is also thought to play as an amino acid sensor for activation of protein kinase A targets. This figure is based on Rubin-Bejerano et al, 2003, Biswas et al., 2003b, Limjidaporn et al., 2003 and Donaton et al., 2003.
nitrogen in the extra cellular medium, \textit{CaGAPI} is induced or repressed. In poor nitrogen source like proline or nitrogen starvation condition, \textit{CaGAPI} is induced by GlcNAc through Cph1p mediated Ras1p signaling pathway, which leads to a morphological change. This interplay between GlcNAc and different nitrogen sources probably brings about a coordinated regulation of \textit{CaGAPI} expression and morphogenesis.

Recently Fink’s group used transcriptional profiling (Microarray) of \textit{S. cerevisiae} and \textit{C. albicans} to analyze the phagosomal microenvironment of the human neutrophils. Their microarray experiments showed that upon ingestion by neutrophils both \textit{S. cerevisiae} and \textit{C. albicans} induces the expression of two above mentioned amino acid regulated genes, general amino acid permease (\textit{GAPI}) and transcription factor \textit{GAT1} (Rubin-Bejerano \textit{et al.}, 2003).